Tesis doctoral

Gestión sostenible de la industria oleícola: co-digestión anaerobia del alperujo con microalgas

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Introducción y objetivos

Introducción

1. El aceite de oliva

La zona mediterránea se cree que fue el centro de origen del olivo salvaje, y por estudios de trazas de polen se conoce su presencia en dicha zona desde hace 3.2 millones de años; aunque la palinología data el origen de *Olea europea* en la era Terciaria. Se sabe que el cultivo del olivo comenzó en la zona de Siria, Líbano, Israel y Turquía unos 4.000 años antes de Cristo (Sánchez-Muniz 2007). Se desconoce la época en que se inicia el cultivo del olivo en España, se acepta que fueron los fenicios o los griegos los que empezaron a cultivar el olivo, pero no fue hasta la época de los romanos cuando el cultivo del olivo se extendió por toda la península. Andalucía ha sido y es la principal tierra de producción de aceite de oliva, siendo Córdoba y Jaén las provincias olivareras por excelencia.

El aceite de oliva es la principal fuente de lípidos de la dieta mediterránea. Está compuesto mayoritariamente de trigliceroles principalmente, ácidos grasos mono y digliceroles. También es rico, en una fracción más pequeña, en compuestos fenólicos de cinco grandes grupos: flavonoides, lignanos, ácidos fenólicos, alcoholes fenólicos y secoiridoides. La composición y concentración en compuestos fenólicos depende de las condiciones

genéticas, agronómicas y tecnológicas de producción y almacenamiento (Melguizo 2019).

La cultura mediterránea ha ido siempre unida al consumo de aceite de oliva (Finicelli et al. 2019), y éste representa la principal grasa consumida por la población de esta región. Hay numerosos estudios que avalan los innumerables beneficios del aceite de oliva. El aceite de oliva posee propiedades nutricionales y nutraceúticas que aporta a las personas que lo consumen, de hecho, los beneficios del aceite de oliva fueron oficialmente reconocidos en el año 2011 por la autoridad Europea del cuidado alimenticio (EASF). Todo esto hace que el consumo de aceite de oliva este en claro aumento, y que cada vez más países comiencen a cultivar y producir aceitunas y aceite de oliva. Aunque a día de hoy, siguen siendo los países del sur de Europa, norte de África y Oriente próximo los principales productores de aceite de oliva. Dentro de estos países cabe destacar a España como el principal productor y exportador de aceite de oliva en el mundo. La producción de la Unión Europea supone el 70% de la producción a nivel mundial, siendo España el principal productor, produciendo un 45% del aceite de oliva Europeo (datos del Ministerio de Agricultura, Pesca y Alimentación, 2019). En España durante la campaña 2018/2019 se produjeron 8.244.187 toneladas de aceitunas destinadas a la elaboración de aceite de oliva, dando lugar a más de 1 millón de toneladas de aceite de oliva virgen (datos del Ministerio de

Agricultura, Pesca y Alimentación, 2019). Además, la producción de aceite de oliva en España se concentra casi de forma exclusiva en territorio andaluz, donde se cultivaron 6.703.275 toneladas de aceitunas destinadas a la elaboración de aceite de oliva; de los 8 millones producidos en España en la última campaña (datos del Ministerio de Agricultura, Pesca y Alimentación, 2019).

1.1. Evolución de sistemas de extracción en España y Andalucía

La esencia del proceso de extracción de aceite de oliva apenas ha cambiado con el paso de los años, básicamente se lleva a cabo la molienda de la aceituna hasta obtener una pasta, de la que se puede extraer posteriormente el aceite por separación, bien por presión o bien por centrifugación, del resto de los componentes de la aceituna. En cambio, la tecnología usada para la extracción del aceite sí ha ido evolucionando poco a poco. El sistema tradicional de extracción de aceite de oliva dio paso en los años 60 al sistema de centrifugación en tres fases y posteriormente evolucionó hasta un sistema de extracción de dos fases en los años 90 del pasado siglo. Sin embargo, esta evolución en el proceso de extracción no ha evitado la generación de subproductos altamente contaminantes.

El sistema tradicional de extracción de aceite de oliva consta de una molienda, donde se produce la pasta de aceituna, un prensado, donde se separa la fase líquida de la sólida y una decantación final

donde se separa el aceite del agua de vegetación. Poco a poco este sistema fue quedando en desuso, ya que requería alto esfuerzo manual y mucho más tiempo de elaboración que los sistemas modernos. Otra razón que contribuyó al detrimento de los sistemas tradicionales fueron la calidad del producto y el control alimentario.

En los años 60 se instauró en España el sistema de extracción en tres fases (Figura 1). Este sistema revolucionó el proceso de elaboración de aceite de oliva, ya que se pasó de un sistema discontinuo basado en el uso de prensas para separar la fase oleosa a un sistema continuo automatizado mediante el uso de una centrífuga horizontal, al que se le unió la incorporación de nueva tecnología como molinos automáticos y decantadores (Cerretani et al., 2009). El sistema de tres fases se expandió rápidamente por los países mediterráneos (80% de las almazaras europeas y la totalidad de las almazaras españolas), debido a su gran capacidad de elaboración de producto y la automatización del proceso, reduciéndose el coste de la mano de obra (Amirante et al., 2010). Este revolucionario sistema por centrifugación en tres fases, permitía separar mediante centrifugación: aceite, alpechín y orujo. El orujo estaba constituido por la parte sólida de la aceituna (restos de huesos, piel y pulpa de aceituna) y el alpechín, por el agua de vegetación que contenía la aceituna, más el agua añadida en los diferentes pasos del proceso de extracción. Finalmente los

alpechines se mezclaban con el agua de lavado de aceituna y de aceite generándose grandes volúmenes de éste agua residual. El alpechín se caracterizaba por ser un subproducto muy rico en materia orgánica con un alto contenido en azúcares, polialcoholes, pectinas, lípidos y compuestos aromáticos que le proporcionaban carácter fitotóxico y antimicrobiano (Muktadirul Bari Chowdhury et al., 2013). En el sistema de tres fases, por cada kg de aceituna procesada, tras el proceso de batido (Figura 1) se le añade al sistema de 80-100 L agua templada, lo que conlleva una dilución de la pasta de aceituna, reduciendo por tanto los antioxidantes naturales presentes, y aumentando considerablemente el volumen de agua residual generada durante el proceso (Amirante et al., 2010).

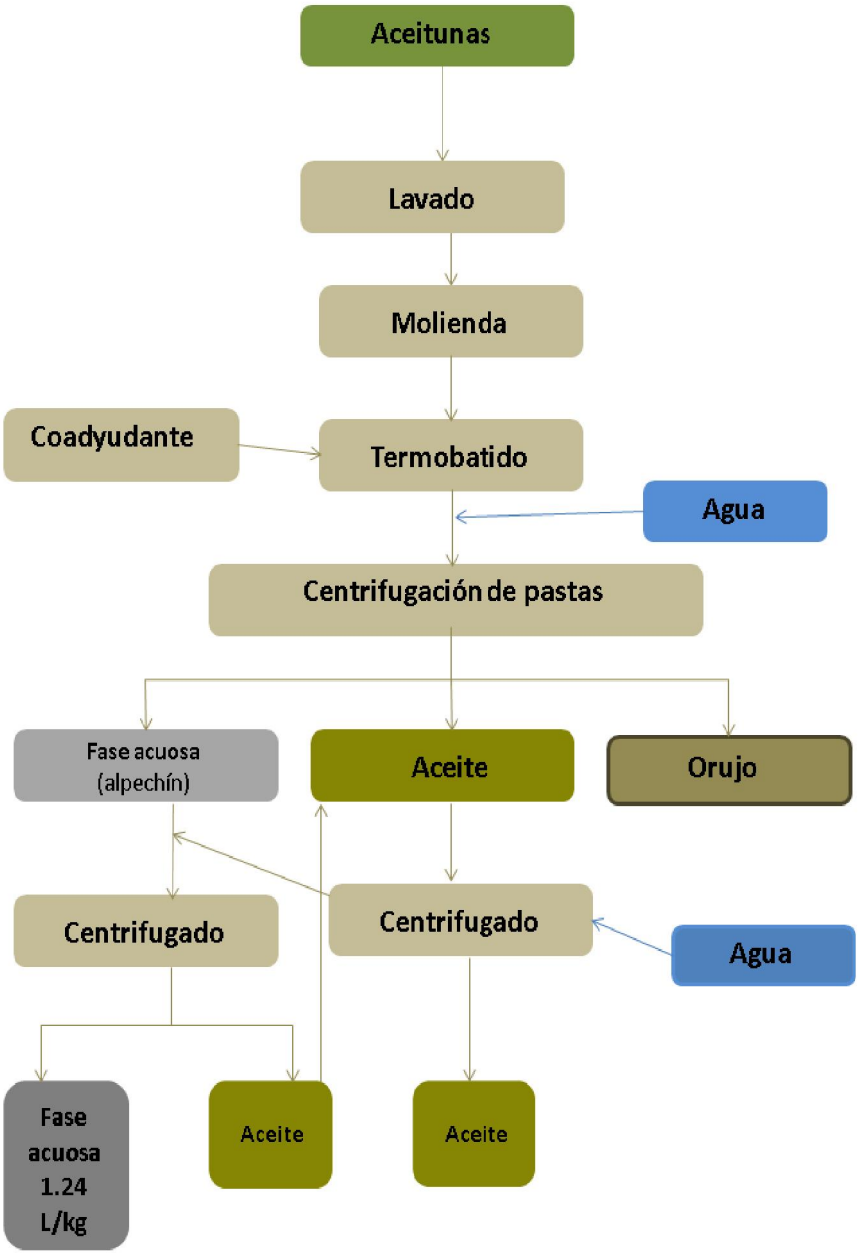

Figura 1. Esquema del proceso de extracción de aceite de oliva en tres fases.

A principios de los años 90, las almazaras españolas empezaron a instaurar un nuevo sistema de extracción de aceite de oliva por centrifugación (Figura 2). El sistema de dos fases aportó a la industria una disminución notoria del uso de agua durante el proceso, una disminución en la producción de efluentes líquidos y un ahorro energético, así como una mejora en la calidad del producto obtenido (Borja et al., 2006). En España este nuevo sistema se instaló como prototipos en algunas almazaras de forma experimental durante la campaña 91/92 y rápidamente se expandió su uso por nuestro país, llegando tan sólo dos años después, a estar instaurado en el 20% de las almazaras españolas (Cerretani et al., 2009). En la actualidad el sistema de dos fases está instaurado prácticamente en la totalidad de las almazaras españolas, mientras que a nivel europeo aún es el sistema de tres fases el que predomina (Cerretani et al., 2009). Se denominó sistema de dos fases porque tras el proceso de centrifugación en dos fases de la pasta de aceitunas se obtenía aceite y un subproducto principal, con un contenido en agua entorno al 65-70%, denominado orujo húmedo o alperujo (Alburquerque et al., 2004). El aceite obtenido por este sistema se caracteriza por tener un mayor contenido en polifenoles y mayor resistencia a la autooxidación, contribuyendo el sistema de centrifugación en dos etapas, en definitiva, a la mejora en la calidad del producto obtenido (Piacquadio et al., 1998).

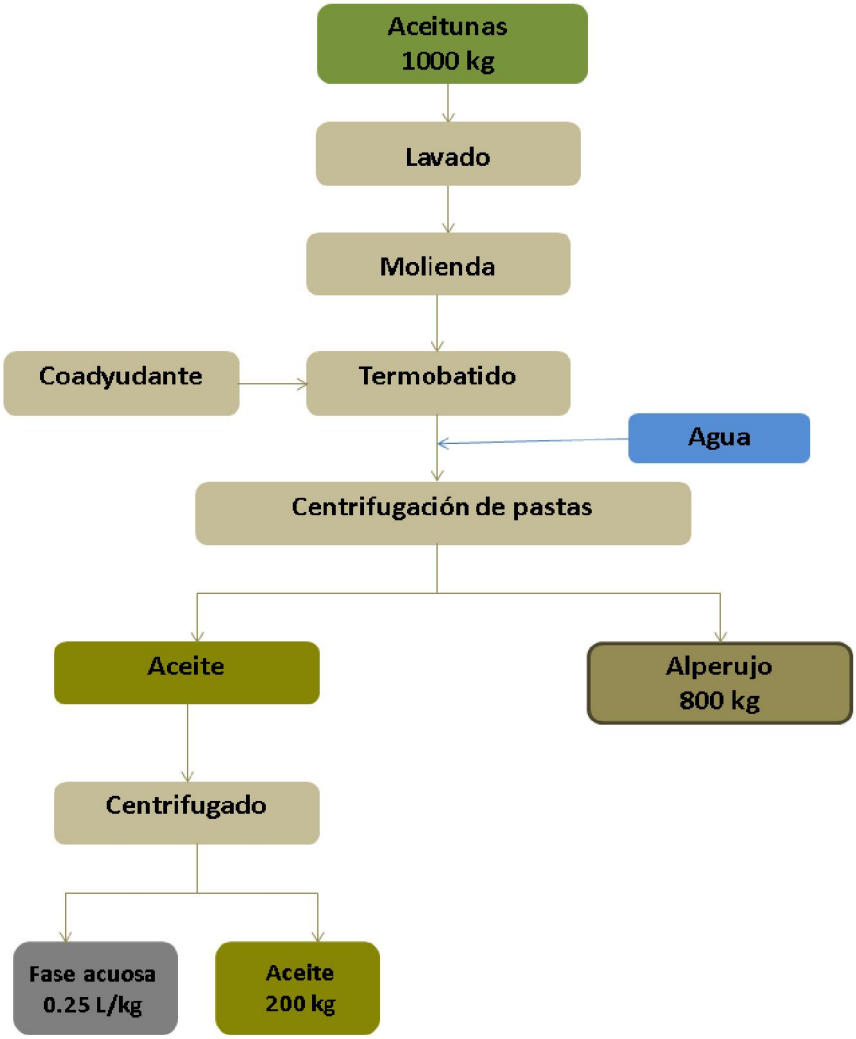

Figura 2. Esquema del proceso de extracción de aceite de oliva en dos fases.

1.2. Producción mundial, producción en España y Andalucía

El aceite de oliva se produce en 56 países de los cinco continentes, incluyéndose en este grupo, además de los productores tradicionales, nuevos países en los últimos años. Según los datos oficiales de los países y las estimaciones de la Secretaría Ejecutiva del International Olive Council (COI), la producción mundial en 2017/18 se estimó en alrededor de 2.854.000 toneladas, lo que supuso un aumento de aproximadamente el 12% en comparación con la campaña anterior. El consumo del aceite de oliva está alcanzando valores de crecimiento récord, sobre todo desde que la dieta mediterránea fuera declarada patrimonio de la humanidad por la UNESCO.

Aunque muchos países están comenzando a producir aceite de oliva, siguen siendo los países Mediterráneos los que concentran el 80% de la producción mundial. España e Italia son los principales productores, mientras que Grecia ostenta la tercera posición a nivel mundial (Carbone et al., 2018).

El aceite de oliva representa uno de los sectores económicos más importantes de nuestro país, sólo durante la campaña 2018/2019 se molturaron un total de 713.810 toneladas de aceitunas (fuente del Ministerio de Agricultura, Pesca y Alimentación, 2019).

Andalucía es la principal productora a nivel nacional de aceitunas para elaboración de aceite de oliva. Sólo en Andalucía se generaron 4.671.729 toneladas de aceitunas durante la campaña 2018/2019 (Datos del Ministerio de Agricultura, Pesca y Alimentación, 2019), que representó el 77,3% de la producción nacional (Figura 3). Siendo las provincias de Jaén, Córdoba y Sevilla las que representaron un 37.8, 28.9 y 12.5%, respectivamente (Datos del Ministerio de Agricultura, Pesca y Alimentación, 2019).

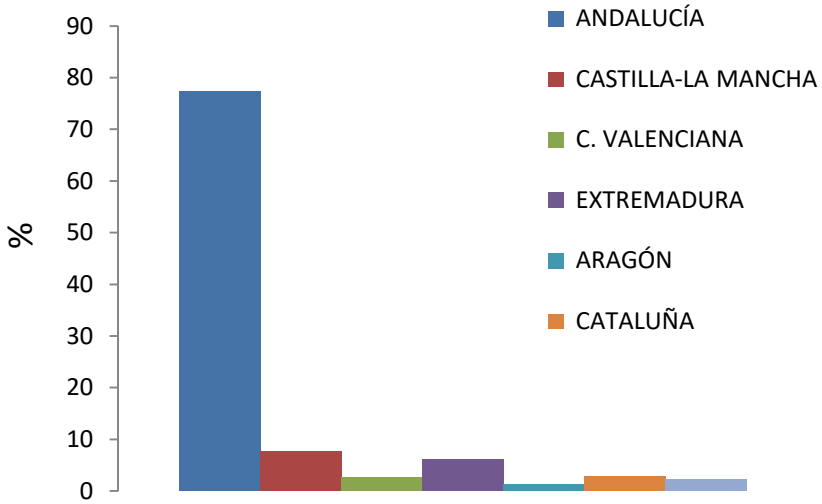

Figura 3. Porcentaje de producción de aceitunas para aceite de oliva en las distintas comunidades autónomas durante la campaña 2018/2019 (Ministerio de Agricultura, Pesca y Alimentación, 2019).

1.3. El alperujo: subproducto de la extracción del aceite de oliva/ problemática medioambiental

A pesar de la gran repercusión económica que genera la industria olivarera, aún hoy en día, el principal subproducto de este proceso causa un gran impacto ambiental en términos de agotamiento de recursos, degradación de la tierra, emisiones al aire y generación de subproductos (Salomone y Ioppolo, 2012). Actualmente el proceso de elaboración de aceite de oliva se ha ido regenerando y el sistema de extracción en dos fases es más sostenible que el de tres fases dada la disminución del uso de agua durante el proceso de elaboración y la contribución a la mejora en la calidad del aceite, si bien, como se ha señalado se genera una gran cantidad de alperujo, su principal subproducto, con un 70% aproximadamente de contenido en agua, además de agua de lavado de aceite y aceitunas (Alburquerque et al., 2004).

Se calcula que, por cada tonelada de aceituna procesada, se generan un total de 800 kg de alperujo y 200 litros de agua de lavado de aceite y lavado de las aceitunas cuando son recepcionadas en la almazara. Se estima una producción de 2.5 a 4 millones de toneladas de alperujo por campaña sólo en España (Ochando-pulido et al., 2013), de las cuales casi un 80% se genera en Andalucía (Ministerio de Agricultura, Pesca y Alimentación, 2019). Además, ésta gran cantidad de subproducto se genera cada año en un muy breve espacio de tiempo (desde noviembre hasta

febrero), lo que dificulta a las almazaras el poder procesar éste residuo sólido de fuerte olor y textura pastosa (Berbel y Posadillo, 2018).

El alperujo es un subproducto de carácter lignocelulósico altamente contaminante, no sólo por la gran cantidad que se genera, sino por su composición, con un alto contenido en Demanda Química de Oxígeno (> 300g O_2/L). Está formado por el hueso de la aceituna, la pulpa y el agua de vegetación. Se trata de un subproducto con pH ácido (5.6), con alta conductividad eléctrica y muy rico en materia orgánica, conteniendo una alta concentración de azúcares, polialcoholes, pectinas, lípidos y compuestos aromáticos que le proporcionan un carácter fitotóxico y antimicrobiano (Muktadirul Bari Chowdhury et al., 2013). Por consiguiente, el manejo inadecuado de este subproducto puede conllevar un impacto medio ambiental realmente importante.

A pesar del gran impacto económico que genera el aceite de oliva en Europa, y de la existencia de normativa a nivel europeo que regula la gestión de los residuos (Directiva 2008/98/CEE de 19 de noviembre, modificada por la Directiva 2018/851/UE, de 30 de mayo), no existe ninguna norma a nivel Europeo que establezca controles para la manipulación y tratamiento de los residuos sólidos y líquidos proveniente de las almazaras. A nivel andaluz, existe el Decreto 4/2011 de 11 de enero, por el que se regula el régimen del uso de efluentes líquidos de extracción de almazara como

fertilizante agrícola, pero no hay ninguna normativa que regule el procesado del alperujo.

En la actualidad, la gran mayoría de las almazaras, van almacenando los alperujos del proceso de elaboración de aceite de oliva en balsas de evaporación, utilizándose parte de los mismos posteriormente para su valorización a escala industrial mediante co-generación para la producción de energía eléctrica. La cogeneración no es una tecnología limpia y produce contaminación por la emisión de humos y cenizas volantes así como óxidos de nitrógeno. Además, la capacidad de las balsas es limitada, por lo que se van construyendo nuevas balsas, conllevando problemas asociados como la posibilidad de desbordamiento, contaminación atmosférica, olores, plagas de insectos, etc. (Karaouzas, 2018).

1.4. Aprovechamiento y tratamientos del alperujo

Debido a la gran cantidad de subproductos que generan las empresas, en los últimos años ha habido una tendencia a revalorizarlos, obteniéndose sustancias de valor añadido, reduciéndose así, la gran cantidad de subproductos generados (Meini et al., 2019, Borges et al., 2019). Esta estrategia de reciclaje no solo evita los costes de eliminación de los subproductos y los problemas ambientales que ocasionan, sino que también aporta un

patrón de valorización para los sectores agrícola y agroindustrial (Muchagato et al. 2018).

El uso de la enorme cantidad de alperujo que se genera en la industria del aceite de oliva para la obtención de compuestos de interés, no sólo eliminaría un problema ambiental, si no que tendría una alta repercusión en la industria alimentaria, cosmética y farmacéutica (Rodrigues et al., 2017). El alperujo está compuesto principalmente por celulosa, hemicelulosa y lignina, pero también contiene grasas, proteínas y una alta concentración de compuestos de valor añadido, como son los polifenoles y polisacáridos (Dermeche et al., 2013). Así, la valorización de los subproductos provenientes del proceso de elaboración de aceite de oliva está en continuo estudio. Montané et al. (2002) estudiaron el uso del alperujo como enmienda orgánica del suelo y la obtención de compost a partir de éste subproducto, pero la emisión de olores y la filtración de agua proveniente del alperujo resultaron problemáticas, haciéndose necesario el uso de biofiltros, suponiendo un incremento notable en los costos de uso de esta tecnología (Kobek 2004). También se ha estudiado el empleo del alperujo para la obtención de furfural y carbón activo (Sánchez et al., 2006), para la obtención de biopolímeros (Ubago-Pérez et al., 2006) o bioetanol, aunque los bajos rendimientos de bioetanol y su bajo poder calorífico (23.4 MJ/kg) lo hacen una alternativa poco viable (Morillo et al., 2009). En el año 2016, Oliveira et al.

estudiaron el uso del alperujo como fuente principal de nutrientes para el crecimiento de hongos del género *Aspergillus* con el fin de producir lipasas. Más recientemente se ha usado el alperujo para la obtención de ácidos grasos volátiles (Cabrera et al. 2019) o para la extracción de fenoles (Rubio-Senent et al., 2017). Hasta la fecha el único estudio de valorización del alperujo llevado a cabo a escala industrial ha sido la co-generación eléctrica, utilizando un sistema de secado previo de este residuo aprovechando el aire caliente que resulta de la condensación del vapor de turbina en un aerocondensador con una eficiencia energética de sólo el 60% (Celma et al., 2008). Debido a que la combustión de éste subproducto es más lenta que la de otros subproductos lignocelulósicos, además requiere una temperatura mayor, más cantidad de aire y un tiempo de residencia más elevado. Todo ésto, junto a la naturaleza del alperujo, que durante el proceso de co-generación emite oxidos de nitrógeno (NO_x) que producen una alta corrosión y un rápido ensuciamiento de los equipos utilizados (Miranda et al., 2008). Durante los últimos años se han disminuido las subvenciones para el uso de biomasa residual con fines energéticos mediante el uso de procesos de combustión, pirólisis o gasificación, por lo que la combustión del alperujo como alternativa para el tratamiento de éste suproducto es cada vez menos rentable. Son necesarios estudios alternativos que lleven a un aprovechamiento y gestión sostenible de unos de los principales subproductosgenerados a nivel español.

2. Digestión anaerobia

La digestión anaerobia se puede considerar como un ecosistema, donde diferentes microorganismos anaerobios realizan la mineralización de la materia orgánica hasta biogás (CH_4, CO_2, H_2, H_2S) mediante complejas y equilibradas interacciones tróficas de forma consecutiva y sinérgica. El biogás que se genera con este proceso biológico, aunque depende del sustrato tratado y del tipo de tecnología utilizada, suele contener entre un 50-70% de metano, 30-40% de anhídrido carbónico, menor de 5% de hidrógeno, ácido sulfhídrico, y otros gases minoritarios (Lettinga et al., 1996). La composición del biogás lo hace susceptible de utilización para aprovechamiento energético mediante su combustión en motores, en turbinas o en calderas, bien de forma individual o mezclado con otros combustibles. El poder calorífico del biogás es de 4.700 a 5.500 kcal/m^3, dependiendo del contenido en metano (Abad et al., 2019).

Actualmente, se tiende hacía la revalorización de los subproductos. La digestión anaerobia es una gran alternativa para la gestión de los subproductos orgánicos procedentes de la ganadería y la agricultura y también es un proceso adecuado para el tratamiento de aguas residuales de alta carga orgánica, como las generadas en muchas industrias alimentarias. La digestión anaerobia es uno de los métodos más sostenibles para el

tratamiento de residuos, ya que presenta numerosas ventajas (Lettinga et al., 1996):

- Baja producción de lodos, ya que los organismos anaerobios se reproducen de 3 a 5 veces más lentos que los organismos aerobios.
- Bajo consumo de energía.
- Alta mineralización de los residuos orgánicos (degradación completa de un compuesto a sus constituyentes minerales, en donde el carbono orgánico es oxidado hasta CO_2).
- No es necesario el uso de mucho terreno para implementar un digestor anaerobio.
- Producción de energía renovable, como resultado del proceso se obtiene metano, con un alto poder calorífico.
- Los microorganismos involucrados en la digestión anaerobia poseen un bajo requerimiento nutricional
- El proceso de digestión anaerobia tolera altas cargas orgánicas.
- Aplicación en pequeña y gran escala.

2.1. Etapas de la digestión anaerobia

El proceso de digestión anaerobia es un proceso complejo, donde las grandes moléculas orgánicas se van degradando hasta metano y anhídrido carbónico principalmente y consta de varias etapas (Figura 4):

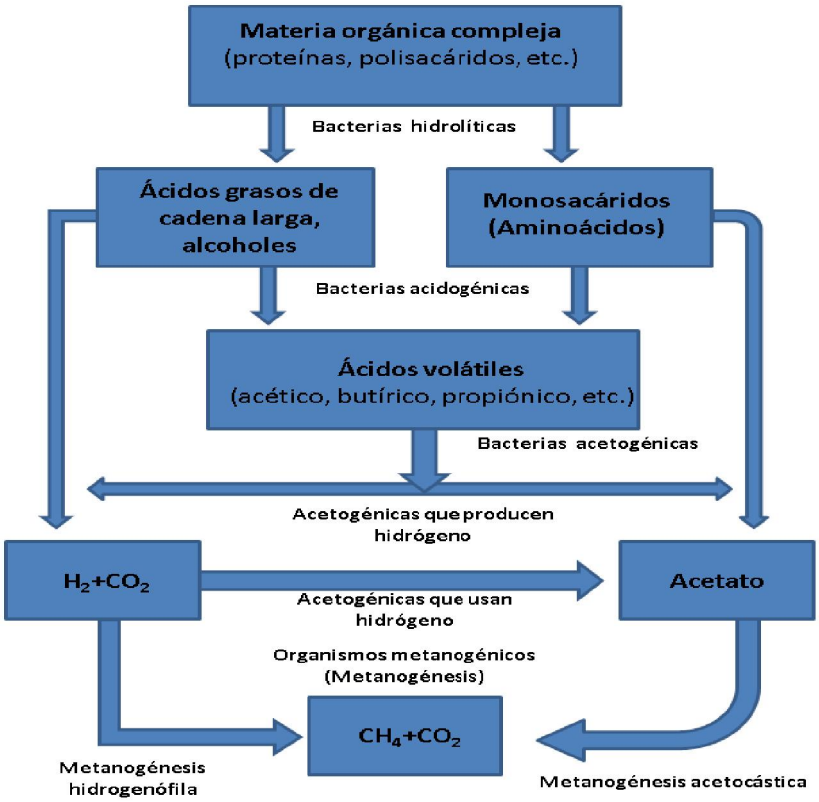

Figura 4. Rutas metabólicas y grupos microbianos involucrados en la digestión anaerobia (Gujer y Zender, 1983).

2.1.1. Hidrólisis y acidogénesis

La hidrólisis es llevada a cabo por microrganismos anaerobios estrictos capaces de romper mediante exoenzimas (celulasas, proteasas, lipasas, amilasas, etc.) la materia orgánica compleja, es decir, proteínas, lípidos y carbohidratos, en monómeros solubles más sencillos como son glucosa, ácidos grasos o aminoácidos. Éstos compuestos simples pueden penetrar a través de las membranas de las bacterias hidrolíticas, donde son metabolizados y convertidos en compuestos más simples que son excretados al medio. Como resultado de este metabolismo se producen ácidos grasos volátiles, alcoholes, ácido láctico, dióxido de carbono, hidrógeno, amonio, sulfuro de hidrógeno, además de nuevas células bacterianas. En esta fase son muy importantes las bacterias del género *Clostridium* y *Staphyloccocus* (Lettinga et al., 1996).

2.1.2. Acetogénesis

Las bacterias acetogénicas parten de los monómeros anteriormente formados y los oxidan hasta ácido acético o fórmico, dióxido de carbono e hidrógeno, que son el substrato apropiado para las arqueas metanogénicas. Durante la formación de ácido acético o propiónico, se forma una gran cantidad de hidrógeno, con lo que conllevaría una bajada de pH. En esta simbiosis, el hidrógeno producido en esta fase bien es consumido por las arqueas

metanogénicas, que usan el hidrógeno y el dióxido de carbono para producir metano, o se consume para formar ácidos como propiónico o butírico. Representantes de los microorganismos acetogénicos son *Syntrophomonas wolfei* y *Syntrophobacter wolini* (Lettinga et al., 1996).

2.1.3. Metanogénesis

Las arqueas metanogénicas son capaces de usar el CO_2, H_2, NH_3 y los ácidos acético y fórmico, siempre en presencia de sales minerales, para la síntesis de sus constituyentes celulares. Además producen metano como producto de desecho. Su tasa de crecimiento es muy lenta y muy dependiente de la temperatura, pero son capaces de producir metano a gran velocidad. Las principales arqueas productoras de metano son del género *Methanosarcina y Methanosaeta* (Lettinga et al., 1996).

Los microorganismos anaerobios en general, se caracterizan por ser sensibles a los cambios del ambiente donde se encuentran, lo que hace importante conocer los parámetros limitantes del proceso y su interrelación.

2.2. Factores que influyen en la digestión anaerobia

Al tratarse de un proceso biológico llevado a cabo por diferentes grupos de microorganismos, el buen difundir del proceso depende de las diferentes velocidades de crecimiento de cada microorganismo y la sensibilidad de éstos a los diferentes compuestos intermedios como H_2, amoniaco, ácido acético, etc. Es importante destacar el tiempo de duplicación de los microorganismos implicados en este proceso; mientras que las bacterias acidogénicas se dividen en 30 minutos, las acetogénicas tardan de 1 a 4 días y las metanogénicas pueden tardar desde 2 hasta 3 días (Gujer y Zender, 1983), siendo éste uno de los factores más importantes para la estabilidad de un digestor anaerobio. Otros factores que afectan al proceso de digestión anaerobia son:

La temperatura: la digestión anaeróbica, al ser un proceso biológico, es muy dependiente de la velocidad de crecimiento de los organismos involucrados y por lo tanto es muy dependiente de la temperatura (Ryue et al., 2019). Al aumentar la temperatura, aumenta la velocidad de crecimiento y por lo tanto aumenta la producción de biogás. En el proceso de digestión anaeróbica existen tres rangos de temperaturas con los que se puede trabajar con los microorganismos anaerobios: psicrófilo (<25ºC), mesófilo (entre 25 y 45ºC), termófilo (entre 45 y 65ºC) y termófilo extremo (>65ºC).

La velocidad del proceso aumenta con la temperatura, pero también aumenta el requerimiento energético y además esta temperatura puede disminuir la estabilidad del proceso (Fannin, 1987). Por el contrario, trabajar a bajas temperaturas, hace aumentar el tiempo de retención hidráulico, por lo que hay que trabajar con reactores más grandes, sin embargo, hay menos problemas de inestabilidad. Por ello la temperatura más usada es la mesófila (25-35 ºC). La temperatura además influye sobre otros parámetros físico-químicos como son la actividad de los microorganismos, la constante de equilibrio de las reacciones químicas, la solubilidad de los gases que se van creando en el proceso y por supuesto en el pH.

pH y alcalinidad: Los cambios de pH influyen principalmente en la actividad enzimática de los microorganismos involucrados en este proceso (Wang et al., 2014). El pH es el parámetro que regula la coexistencia de las diferentes poblaciones microbianas. Para que el proceso se desarrolle bien, el pH tiene que estar próximo a la neutralidad, no debiendo nunca bajar de 6.5 ni subir de 8.3 (Yuan et al., 2016). Si el pH es inferior a 6.5, se produce una bajada en el consumo de los ácidos orgánicos que se acumulan provocando un decrecimiento adicional del pH que impediría que el proceso continúe hasta la producción de metano. Además el pH es un controlador del sistema, ya que interviene en diferentes equilibrios químicos, y pudiendo dar lugar a la formación de determinados productos con influencia sobre el sistema. Por ejemplo, el pH juega

un papel importantísimo en el equilibrio amonio-amoniaco y por lo tanto en el proceso de digestión anaerobia en general, ya que al amoniaco libre es uno de los inhibidores más importantes de la fase metanogénica (Yen y Brune, 2007).

La alcalinidad no es más que una medida de la capacidad que tiene un sistema para neutralizar ácidos. En el rango de pH de 6 a 8, el principal equilibrio químico que controla la alcalinidad es el dióxido de carbono-bicarbonato. La alcalinidad al bicarbonato debe mantenerse por encima de 2.500 mg/L para asegurar la estabilidad del digestor (Fannin, 1987).

Sustancias inhibitorias: La digestión anaeróbica también puede ser inhibida por compuestos que afecten a los microorganismos tales como los fenoles (Rubio-Senent et al., 2017) o por la presencia de ácidos (Chen et al., 2008)

Relación Carbono/Nitrógeno (C/N): se ha demostrado que otro factor muy importante a tener en cuenta durante la digestión anaeróbica es la relación C/N, siendo las relaciones C/N próximas a 30/1 las más indicadas para el buen desarrollo de la digestión anaerobia (Paul y Dutta, 2018). Bajos niveles de nitrógeno (alta relación C/N) son característicos de sustratos de bajo pH y poca capacidad tampón, en la digestión de este tipo de sustratos una acumulación de ácidos grasos volátiles daría lugar a una rápida desestabilización del proceso de digestión debido a la baja

concentración de alcalinidad y la baja capacidad tampón (Paul y Dutta, 2018), por el contrario, una baja relación C/N, provocaría en el sistema una alta concentración de amonio, que probablemente sería mayor que la cantidad necesaria para el crecimiento bacteriano, con lo cual podría llegar a darse una inhibición del proceso de digestión igualmente (Paul y Dutta 2018). Valores de amonio entre 1.700-1.800 mg/L se han descrito en la literatura como tóxicos para el proceso de digestión anaerobia (Yenigün y Demirel 2013).

La relación C/N del alperujo está por encima del valor indicado por Paul y Dutta (2018) como óptimo para la digestión anaerobia, además de la alta concentración de polifenoles presente en el alperujo, son dos factores que limitan la digestión anaerobia. El uso de un co-sustrato rico en nitrógeno, ayudaría a balancear la relación C/N hacia los valores indicados como óptimo, y ayudaría a diluir la concentración de sustancias tóxicas que posee el alperujo (Li et al., 2018; Ferreira et al., 2018).

3. Características fisiológicas y morfológicas de las microalgas

Las microalgas son microorganismos unicelulares fotosintéticos, autótrofos o heterótrofos, que crecen en ecosistemas de agua dulce o salada. Se caracterizan por ser unas eficientes fijadoras de CO_2,

utilizando la energía proveniente de la luz solar para producir biomasa (Mata et al., 2010).

Dentro de las microalgas se engloban organismos filogenéticamente muy diversos, desde procariotas capaces de realizas fotosíntesis oxigénica, como las cianobacterias y las proclorofitas, hasta organismos eucariotas (Mata et al., 2010). Esta diversidad genética conlleva una diversidad metabólica y bioquímica, que les confiere un amplio rango de propiedades y de características que les permite vivir en ecosistemas muy diversos. La composición bioquímica de las microalgas (contenido en lípidos, carbohidratos y proteínas) es bastante variable dependiendo de la especie, además, esta composición puede ser modificada por las condiciones de cultivo (Ling et al., 2019).

La enorme versatilidad de las microalgas, junto a su alta tasa de crecimiento, les confiere una gran importancia a nivel industrial (Morales-Sánchez et al., 2017). Otra ventaja de las microalgas es que pueden crecer en tierras pocos productivos a gran escala, tanto en agua limpias como en aguas residuales (Mata et al., 2010). A nivel biotecnológico el interés sobre las microalgas está en auge, ya que tienen un gran potencial para aplicaciones medioambientales y como productoras de compuesto de valor añadido (Mata et al., 2010).

Hoy en día las microalgas también son consideradas como una alternativa para la obtención de energía renovable, y la principal dificultad a resolver es el de los costes de producción (Frac et al., 2010). Así son consideradas como una de las principales fuentes de energía alternativa, ya que pueden crecer en el mar, y en tierras no cultivables, además de ser capaces de acumular una gran cantidad de lípidos, proteínas y otras sustancias de gran interés (Mata et al., 2010).

3.1. Obtención de biomasa algal en aguas residuales

El cultivo de las microalgas en aguas residuales es una opción cada vez más viable que permite bajar los costes de producción del cultivo (Rayen et al., 2019). Como ventaja de este crecimiento, no sólo se obtiene una gran cantidad de biomasa, si no que las microalgas ayudan a eliminar nutrientes de éste tipo de aguas, principalmente nitrógeno y fósforo, ayudando a la biorremediación, y evitando la eutrofización de los ecosistemas acuáticos naturales (Leite et al., 2019).

El tratamiento de las aguas residuales con microalgas es un proceso aerobio de bajo coste, ya que no necesita aireación, y es el oxígeno producido por los propios organismos fotosintéticos el que es posteriormente usado por las bacterias aerobias para la oxidación de la materia orgánica y el ión amonio (Huang et al., 2015).

Además, el crecimiento autótrofo y heterótrofo de las microalgas favorece la eliminación de contaminantes, nutrientes e incluso metales pesados (Matamoros et al., 2016; Jais et al., 2017).

El crecimiento de microalgas en aguas residuales y usando la energía del sol para su crecimiento en consorcio con bacterias, es la alternativa más rentable a la producción de microalgas a gran escala (Mata et al., 2014)

3.2. Valorización de las microalgas

El interés comercial de las microalgas se debe principalmente a su capacidad de acumular compuestos de valor añadido (Kothari et al., 2017). Existen numerosos estudios sobre la acumulación de lípidos en microalgas (Bian et al., 2018; Shomal et al., 2019), muchos de ellos relacionados con la generación de biodiesel, o la acumulación de carbohidratos para la obtención de bioetanol (Ngamsirisomsakul et al., 2019). Aunque el coste de producción de la biomasa, junto al elevado coste de la extracción de los lípidos y carbohidratos de las algas, hoy en día por la falta de conocimiento tecnológico, lo hace una alternativa poco viable (Bian et al., 2018). También se usan las microalgas para la obtención de carotenoides (Di Lena et al., 2019) como astaxantina (Ledda et al., 2016), que se acumula en grandes cantidades en el género *Haematococcus* sp., o ß-caroteno que se acumula

principalmente en *Dunaliella salina* (Han et al., 2019). La obtención de proteínas a partir del cultivo de microalgas y el modo de extraerlo, es un tema que está en continuo estudio (Chew et al., 2019). Hernández-García et al. (2019), estudió la acumulación de lípidos y carbohidratos en las microalgas *Desmodesmus* spp. y *Scenedesmus obliquus* crecidas en un consorcio con bacterias en aguas residuales de lixiviado, obteniendo una concentración de lípidos del 20%, con una alta concentración de ácidos grasos insaturados y hasta un 41% de carbohidratos; consiguiendo una gran eliminación de nutrientes de las aguas (82% amonio y 43% de ortofosfato). La recuperación de fósforo de las microalgas para su posterior uso como fertilizantes también es un tema de bastante interés (Huysman et al., 2019). También resulta muy interesante el uso de las microalgas para la fijación de CO_2 y por lo tanto, la reducción de gases de efecto invernadero (Cheng et al., 2019). El uso de la biomasa algal para la producción de biogás durante la digestión anaerobia (Perendeci et al., 2019) es otra de las alternativas estudiadas, aunque en estudios recientes se ha demostrado que la pared celular de las microalgas puede limitar la fase hidrolítica de la digestión anaerobia (Jankowska et al., 2017), y por lo tanto su producción de metano. En cambio, ensayos de co-digestión anaerobia, donde dos o más sustratos son co-digeridos en un reactor anaerobio, han demostrado una mejora en la actividad enzimática y en la rotura de la pared celular (Zhang et al., 2019).

4. Beneficios de la co-digestión anaerobia: alperujo/microalga

Hay numerosos estudios de investigación que acreditan las ventajas del proceso de co-digestión anaerobia; incluido el aumento en la producción de biogás. Hartmann y Ahring, (2005) resaltaron como beneficios el balance de la relación C/N, así como la concentración de macro y micronutrientes, pH y compuestos complejos e inhibitorios de la digestión anaerobia. Años más tarde, Ajeej et al. (2015) observaron cómo entre los beneficios de la co-digestión anaerobia, el incremento de la actividad metanogénica de las arqueas, una disminución en la inhibición por amonio e incluso un aumento en la actividad hidrolítica de las bacterias.

Los subproductos provenientes de la agricultura, y en concreto el alperujo, se caracterizan por ser un subproducto de composición lignocelulósica muy compleja, con una alta relación C/N, una gran cantidad de sustancias inhibitorias para el proceso de digestión anaerobia y un pH ácido; por lo que es necesario buscar un sustrato con una alta concentración de nitrógeno para poder usarlo como co-sustrato, balancear la relación C/N y diluir la presencia de sustancias inhibitorias para el proceso de digestión anaerobia.

Las microalgas tienen un alto potencial, como biomasa rica en nitrógeno, de ser usada como co-sustrato de subproductos ricos

en carbono, teniendo en cuenta que la relación C/N de las microalgas suele estar en torno a 10/1 (Geider y La Roche, 2002). Además, la posibilidad de hacer crecer la biomasa algal en aguas residuales le añade aún más valor a este tipo de estudios.

5. Aplicaciones posteriores del digestato procedente de la digestión anaerobia

Como resultado del proceso de digestión anaerobia se genera lo que se denomina digestato. Se trata de un producto semi-estabilizado, con un alto contenido en agua. Tras la digestión anaerobia, tanto el nitrógeno como el fósforo pasan de estar en forma orgánica a mineral, pero no se eliminan del sistema, por lo que el digestato se caracteriza por ser rico en nitrógeno y fósforo, de ahí su gran potencial para uso como abono agrícola o para la fabricación de fertilizantes (O'Brien et al., 2019).

Hasta la fecha hay numerosas investigaciones sobre el potencial uso del digestato como fertilizante agrícola, aunque en investigaciones llevadas a cabo en el año 2015 (Dahlin et al., 2015) se observó que un uso prolongado del digestato en un mismo suelo puede llevar a una sobre carga de nutrientes en dicho suelo y los consiguientes problemas medioambientales (Dahlin et al., 2015). En cambio en un estudio reciente Verdi et al. (2019) definen el uso del digestato como fertilizante como una gran oportunidad para

reducir el impacto ambiental derivado de la fertilización mineral. La calidad y características del digestato obtenido dependen del substrato empleado en la digestión anaerobia. Actualmente, la mayoría de los estudios son realizados con digestatos procedentes de la digestión anaerobia de excrementos animales (Bustamante et al., 2019; O´Brien et al., 2019; Montemayor et al., 2019), con una gran concentración de nitrógeno y en los que se hace necesario pasar por un proceso de esterilización o pausterización para eliminar la presencia de patógenos. En cambio, existen pocos estudios sobre el uso del digestato proveniente de la digestión anaerobia de sustratos vegetales como el alperujo.

El uso eficaz del digestato sumaría otro valor añadido a la digestión anaerobia y permitiría un manejo aún más sostenible de los subproductos. Hasta la fecha el uso del digestato sigue sin ser explotado de manera eficiente y es necesario seguir investigando.

6. Balance energético positivo y producción de energía proveniente de fuentes renovables

En el año 2015 la Unión Europea acordó una serie de directrices, y recientemente han sido actualizadas en marzo de 2019 con un plan de refuerzo de la economía circular donde la reducción, reutilización y reciclado son los principales ejes de la economía, a fin de reducir la producción de residuos y utilizarlos como recursos

(EC, 2015). El concepto de economía circular junto a economía verde (EAA, 2013) y bioeconomia (renovada en 2018) (EC, 2012, 2018), son el centro de la discusión actual de la política internacional, cuyos objetivos son proponer soluciones para conciliar objetivos económicos, ambientales y sociales (D'Amato et al., 2019). Este tipo de economía no se pretende usar sólo a nivel personal, sino que se busca tanto a nivel micro (producto, empresas, consumidores), meso (parques ecoindustriales) y macro escala (ciudad, región, etc) (D'Amato et al., 2019). Los subproductos industriales reúnen las características para ser objetivos claves en esta estrategia de economía circular.

La digestión anaerobia es una de las herramientas más indicadas para el tratamiento de los subproductos orgánicos, eliminado materia orgánica y por lo tanto carga contaminante de este tipo de subproductos; y obteniendo como resultado un biogás con una concentración de metano dependiente del substrato usado y con un poder calorífico de entre 4.700 a 5.500 kcal/m^3 (Abad et al., 2019).

La digestión anaerobia del alperujo es un proceso bastante estudiado, observándose como las sustancias inhibitorias como los fenoles, limitan la producción de metano (Borja et al., 2003; Rincón et al., 2016; de la Lama et al., 2017). Además de la compleja estructura lignocelulósica del residuo, que limita la fase hidrolítica de la digestión anaerobia y por tanto la producción de

metano (Hendriks y Zeeman, 2009). El uso de pre-tratamientos para sustratos complejos como el alperujo, mejoraría la producción de metano (Hendriks y Zeeman, 2009; Rincón et al., 2016; de la Lama et al., 2017), pero la inversión energética de los pre-tratamientos no recompensaría la producción de energía (Fan et al., 2017). En cambio, la co-digestión anaerobia de dos o más sustratos, es la opción de la digestión anaerobia más rentable (Gandiglio et al., 2017). El uso de un co-sustrato, no sólo balancea la relación C/N, si no que diluye la concentración de sustancias inhibitorias en el reactor y mejora la actividad hidrolítica de las bacterias (Barua et al. 2018). En el caso de subproductos lignocelulósicos ricos en C, el uso de un sustrato rico en nitrógeno como son las microalgas, que pueden crecer en aguas residuales y con energía solar, hacen la co-digestión anaerobia de este tipo de subproductos con microalgas, la alternativa más viable, siguiendo las indicaciones de la Economía Circular dictada por la Unión Europea en el año 2015 y reforzada en marzo de 2019. Además, el uso del digestato resultante de ésta co-digestión como enmienda orgánica, sumaría una rentabilidad extra al biogás y por ende al proceso de digestión anaerobia.

7. Objetivos

Este trabajo de investigación, realizado en el marco del Proyecto de Excelencia de la Junta de Andalucía RNM-1970: "Gestión sostenible de la industria oleícola: co-digestión anaerobia del alperujo con microalgas, valorización del biogás y los efluentes obtenidos" tiene como propósito central el tratamiento del subproducto semisólido proveniente del proceso de elaboración de aceite de oliva por centrifugación en dos fases, orujo húmedo o "alperujo", mediante su co-digestión anaerobia con microalgas. Para conseguir este propósito se han estudiado los siguientes objetivos concretos:

1. Evaluación de la influencia de un pre-tratamiento hidrotérmico suave sobre el alperujo, estudiando la influencia de la temperatura, el tiempo y la presión en la composición final del alperujo y el efecto que este pre-tratamiento tiene en la digestión anaerobia del alperujo.

2. Estudio de la co-digestión anaerobia del alperujo con diferentes tipos de microalgas: *Scenedesmus*, *Chlamydomonas reinhardtii*, *Dunaliella salina* y *Raphidocelis subcapitata* para mejorar la alta relación C/N del alperujo y optimizar la producción de metano obtenida durante la digestión anaerobia del mismo.

3. Estudio de la influencia de la pared celular de las microalgas en la digestión anaerobia y en la co-digestión anaerobia del alperujo con la microalga *Chlamydomonas reinhardtii*.

4. Estudio comparativo de la evolución de los principales parámetros de la digestión anaerobia durante el transcurso de los experimentos de determinación del potencial bioquímico de metano (BMP) del alperujo, del alperujo pretratado hidrotérmicamente y de la co-digestión anaerobia alperujo-microalga *Dunaliella salina*.

5. Estudio de las cinéticas de producción de metano observadas durante los ensayos de BMP del alperujo pretratado y co-digerido con diferentes microalgas.

6. Estudio del crecimiento de microalgas en las aguas residuales provenientes del proceso de elaboración de aceite de oliva por centrifugación en dos fases e influencia de la co-digestión anaerobia de estas microalgas crecidas en aguas residuales con alperujo mediante ensayos de BMP.

7. Estudio de la cinética de eliminación de nutrientes en las aguas provenientes del proceso de elaboración de aceite de

oliva por centrifugación en dos fases tras el crecimiento de la microalga *Raphidocelis subcapitata*.

8. Estudio del uso del alperujo y del digestato resultante de la digestión anaerobia del alperujo como enmienda orgánica y fertilizante para el crecimiento de la planta forrajera *Lolium rigidum*, destinadas a la a alimentación animal.

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Chapter 1

Impact of an autoclaving soft-hydrothermal pre-treatment in the olive mill solid waste. Change in the Physicochemical characteristics and influence on the subsequent anaerobic digestion

This chapter has been submitted as:

M.J. Fernández-Rodríguez; D. De la Lama-Calvente; A. Jiménez-Rodríguez; R. Pino-Mejías; R. Borja; B. Rincón-Llorente. Impact of an autoclaving soft-hydrothermal pre-treatment in the olive mill solid waste. Change in the Physicochemical characteristics and influence on the subsequent anaerobic digestion

Abstract

The aim of this study was to investigate the effect of a soft-hydrothermal pre-treatment (SHP) on olive mill solid waste (OMSW) and its subsequent anaerobic digestion (AD). OMSW was pre-treated in an autoclave at temperature (121 ºC and 133 ºC) and pressure (1.1 and 2.1 bar, respectively) at heating times of 15, 20 and 30 minutes. An important solubilisation of high valuable compounds such us hydroxytyrosol, and 3,4-dihydroxyphenylglycol was observed after pre-treatments. SHP showed a significant reduction on fiber length and width. A higher polysaccharides solubilisation was observed in treatment at 121 ºC comparing with that observed at 133 ºC. SHP carried out at 121 ºC, 1.1 bar (30 min) (pre-treatment A1), allowed obtaining the highest methane yield (380 ± 5 mL CH_4/g VS), which was 12.3% higher than that obtained for untreated OMSW. Pearson correlation (PEC) and Principal Component Analysis (PCA) were carried out. PEC showed a positive correlation with phenol vanillic acid and PCA grouped pre-treatment A1 with polysaccharides solubilization. The influence of the SHP conditions on the AD of OMSW was assessed through the monitoring of process performance and calculation of kinetic parameters by using the Transference Function model.

1. Introduction

98% of the olive cultivation areas are located in Mediterranean zones which produce 97% of the olive oil in the world. In the 2016-2017 season, the worldwide olive oil production was 2,586,500 tons, 44% of this production came from Spain (AICA, 2016; COI, 2018).

The two-phase olive mill solid waste (OMSW) is mainly composed of water (60-70%), lignin (13-15%), cellulose and hemicellulose (18-20%), olive oil retained in the pulp (2.5-3%) and mineral solids (2.5%), which result in an elevated polluting load with a chemical oxygen demand (COD) in the range of 300-350 g O_2/kg (Rincón et al., 2013).

The anaerobic digestion (AD) of lignocellulosic wastes has been proven to be a more convenient and feasible option compared to other treatments such as physical, physicochemical or biological aerobic treatments due to: a) a high degree of purification can be achieved with high-organic-load feeds; b) low nutrient requirements; c) small quantities of excess sludge are produced; d) a combustible biogas enables the process to generate energy (Motte et al., 2014). Thus, AD has been proposed for the treatment of OMSW, and results show that this waste is anaerobically biodegradable at a mesophilic temperature with a COD removal efficiency in the range of 96.8% - 82.9% (Borja et al., 2006).

The AD process occurs in a sequence of four biological steps, c.a. hydrolysis, acidogenesis, acetogenesis and methanogenesis.

Generally, the hydrolysis is the rate-limiting step, where hydrolytical bacteria release extracellular enzymes which break down organic particulate matter and allow it to solubilise (Nguyen et al., 2018).

Thermal, thermo-chemical and enzymatic pre-treatments, two-stage AD, composting, ensiling and mechanical treatments have been studied in order to enhance the methane production of lignocellulosic biomass by reducing the hydrolytic step (Carrere et al., 2016; Duque et al., 2017). Carrere et al. (2016) concluded that the best treatment for lignocellulosic biomass first requires delignification, followed by hemicellulose and cellulose alkali or biological hydrolysis, although there is no mention of hydrothermal pre-treatments.

Hydrothermal pre-treatment by Liquid Hot Water (LHW), consisted of biomass pre-treated at 120 ºC to 260 ºC and 1-3.5MPa several minutes and diluted in water. This pre-treatment enhanced the dissociation of water molecules which act as an acidic catalyst. LHW eliminates problems with corrosion and operational costs, which are particularly reduced when compared to enzymatic and chemical treatments (Garrote et al., 1999; Ziemiński et al., 2014). LHW pre-treatment (at 160 ºC) enhanced the methane production yield by 76% when using sugar beet pulp as substrate (Ziemiński et al., 2014). Dos Santos Rocha et al. (2017) showed that LHW (195 ºC/15 min) pre-treatment of sugarcane straw reached 85% and 21% of hemicellulose and cellulose removal, respectively. Abu Tayeh et

al. (2016) showed that LHW pre-treatment combined with C1-C2 organic acids improved enzymatic saccharification of OMSW at mild temperatures and pressure (120, 140 and 170 ºC). Jia et al. (2017) showed that short-term hydrothermal pre-treatment (STH) (50%dilution in water and treatment at 90 ºC,30 min) of food waste before two-stage AD enhances the production of biogas when comparing with the process without pre-treatment or with one-stage AD. In this case, the maximum biogas production rate (R_{max}) reached was improved by 59% in the hydrolytic step and by 5% in the methanogenic step.

Another type of hydrothermal pre-treatment is autoclaving. This method consists of high pressure sterilization of waste by steam, which cooks the waste and destroys most of the bacteria in it. Temperature and time usually range between 120 ºC and 160 ºC within 1h (Ibrahim et al., 2011). Pressure usually ranges between 1 and 15 bars. Most of the published results show an increase in methane production when compared to untreated substrate (Pecorini et al., 2016). Bougrier et al. (2008) reported that the use of higher temperatures (> 180 ºC) decreased the biodegradability of the wastes and biogas production. As a special case, the soft-hydrothermal pre-treatment (SHP) in this study consisted of the autoclaving of OMSW at low temperatures (120 ºC – 130 ºC) and pressures (1-2 bar).

Thermal pre-treatments have the disadvantage of releasing soluble-sugar-derived by-products such as furfural, 5-

hydroxymethyl furfural (5-HMF), or lignin-derived by-products such as vanillin, syringaldehyde and other phenolic compounds (Monlau et al., 2012). Their concentrations and nature depend mainly on the biomass origin, but also on the kind of pre-treatment, contact time, pH, pressure, temperature, concentration and solid loading (Mussatto and Roberto, 2004). These compounds have shown an inhibitory effect in several processes such ethanol fermentation, xylitol and butanol production, enzymatic hydrolysis, bio-hydrogen production and in mixed cultures (Quéméneur et al., 2012). Monlau et al. (2014) reviewed the literature data on the impact of pre-treatment by-products on AD processes when using mixed cultures as inoculum and concluded that no minimal inhibitory concentration of each by-product has been successfully found nor the synergistic effect between different by-products.

The aim of the present study was to assess the effect of a SHP on the chemical composition of OMSW using temperatures of 121 ºC and 133 ºC and pressures of 1.1 and 2.1 bar, respectively, at heating times of 30, 20 and 15 min for each treatment. Soluble COD (sCOD), phenol composition, sugar and fiber length were determined after pre-treatment. The digestibility of pre-treated OMSW compared to untreated OMSW was determined in terms of methane potential through biochemical methane potential (BMP) tests. Kinetic modelling of the BMP assays was also performed. The principal component analysis (PCA) and Pearson correlation

(PEC) were applied in order to comprehend how affected the different SHPs to methane production.

2. Materials and methods

2.1. OMSW

The two-phase OMSW was collected from the Experimental Olive Oil Mill Factory (Instituto de la Grasa (CSIC), Seville,Spain). In order to remove olive stone pieces, the OMSW was sifted through a 2 mm mesh.

2.2. SHP

Six different SHPs were carried out on OMSW in an autoclave. The pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively). The pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively). These temperatures were chosen based on previous results obtained on thermal pre-treatment for OMSW and for other lignocellulosic biomasses (Ibrahim et al., 2011; Pecorini et al., 2016; Rincón et al., 2013). 500 g of OMSW were introduced into a 1L autoclavable bottle for each pre-treatment and then autoclaved under the different selected conditions. The samples were stored at 4 ºC (less than 24 h until use).

2.3. Analytical methods and equipment

The pre-treatments were carried out in an autoclave Raypa RFG. The untreated and pre-treated OMSWs as well as the anaerobic digestates after the ADwere analyzed. All substrates were characterized by the measurement of total COD (tCOD) (Raposo et al., 2008). Volatile solids (VS) were determined according to the standard methods 2540E. Total alkalinity was analyzed by pH titration (pH-meter model Crison 2.0 Basic) (APHA, 2005). Fiber viscosity was determined according to Norm UNE-EN-ISO 5351:2004 (Norm UNE-EN-ISO 5351:2004). Total oil content was determined by the Soxhtlet extraction method (NORM UNE 55-032-073). The oil fraction was analyzed by high pressure size exclusion chromatography to determine the content of triglycerides, diglycerides and fatty acids according to the IUPAC Standard Method 2.508 (IUPAC, 1992).

Soluble parameters were determined after sample centrifugation (7500 rpm, 8 min) and two filtrations (filter paper and glass fiber filter). sCOD was determined by a closed digestion and the standard method 5220D (APHA, 2005). Soluble total phenolic content was determined by the Folin-Ciocalteu method (Folin and Ciocalteu, 1927), while total carbon (TC), total organic carbon (TOC) and total nitrogen (TN) were determined using a total organic carbon analyzer (TOC-5000A; Shimadzu Corp.). In order to obtain a distribution of the untreated and pre-treated OMSW fiber lengths and diameters, the matrix was dissolved using a

Soxhlet apparatus and decalin as solvent. Untreated and pre-treated OMSW were as previously filtered using a cellulose filter and set into the Soxhelt equipment (48 h). The obtained fibers were rinsed with acetone and distilled water, the fibers were dried in an oven at 105 °C (24 h).

The length and diameter distributions of the fiber were characterized using a Morfological fiber analyzer (Techpap SAS, France).

2.4. Analysis of individual compounds

Individual phenols and acetic acid were quantified using a Hewlett-Packard 1100 liquid chromatography system using a diode array detector with Rheodyne injection valves (loop of 20 mL) and quantification wavelengths of 254, 280 and 340 nm. A C18 column (250 mm x 4.6 mm internal, diameter 5 mm) was used. Milli Q water acidified (0.01 % trichloroacetic acid and acetonitrile) was used as mobile phase. The gradient applied was 95% at the beginning, 75% in 30 min, 50% in 45 min, 0% in 47 min, 75% in 95 min and 95% in 52 min, being the total run time of 55 min.

The soluble polysaccharide composition was determined by acid hydrolysis with 2 N trifluoroacetic acid (121 ºC, 1 h) (Ruiter and Burns, 1927), derivatization to alditol acetates and quantification by gas chromatography. The soluble monosaccharide composition was quantified by gas chromatography (Englyst and Cummings, 1984).

A HP 6890 Plus+ gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a 30 m x 250 μm x 0.20 mm capillary column (SP-2330, Supelco, Bellefonte, PA) was used. The carrier gas was helium (constant flow of 2.2 mL/min and 21.5 psi). Injection was performed in splitless mode. The oven temperature was held at 50 ºC for 2 min after injection, then programmed to 180 at 35 ºC/min, held at 180 ºC for 5 min, and then immediately increased to 220 at 5 ºC/min, and held at 220 ºC for 22 min. Total run was 40.7 min. The injector temperature was 250 ºC, flame ionization detector (FID), 300 ºC. Neutral sugars, L-rhamnose, D-fucose, L-arabinose, D-xylose, D-mannose (Man), D-galactose and D-glucose were identified. *myo*-Inositol was used as internal standard.

The glucose in the hydrolysates was quantified by the anthrone assay (Dische, 1962). The absorbance values of the standards and samples were measured at 630 nm in a microplate reader (MPM 600; Bio-Rad Laboratories, Inc., Hercules, CA).

2.5. Inoculum for AD

The anaerobic sludge used as inoculum in the reactors was collected from an industrial up-flow anaerobic sludge blanket reactor which treats brewery wastewater in Seville (Spain). The main characteristics of the inoculum used were: pH: 6.77; TS: 28.7±2.7 g/kg; VS: 22.8±2.3 g/kg.

2.6. BMP tests

The BMP tests were carried out in a thermostatic bath at mesophilic temperature (35±2 ºC). Each reactor had an effective volume of 250 mL and was continuously stirred with magnetic bars (450 rpm). The inoculum/substrate ratio was 2 (on a VS basis). For each reactor containing 210 mL of inoculum, the amount of substrate needed to give the required inoculum to substrate ratio was added together with trace element solution (Rincón et al., 2013). Two reactors with the inoculum and trace element solution (without substrate addition) were used as controls.

The reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 3N NaOH solution to capture CO_2; the remaining gas was assumed to be methane.

Seven different substrates (untreated and pre-treated OMSW at different conditions: A1, A2, A3, B1, B2 and B3) were digested in order to show the effect of pre-treatment on the AD. The AD experiments were run in batch mode for a period c.a. of 30 days until the accumulated gas production remained unchanged, i.e. on the last day production was lower than 2% of the accumulated methane produced. Each experiment was carried out in triplicate.

2.7. Kinetic study

The Transference Function (TF) model was applied to fit the experimental data of methane production during BMP tests (eq. 1) (Donoso-Bravo et al., 2010; Li et al., 2012; Serrano et al., 2017):

$$B = B_{max} * \left(1 - exp\left[-\frac{R_{max}(t-\gamma)}{B_{max}}\right]\right) \quad (1)$$

Where B (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, R_{max} is the maximum methane production rate (mL CH_4/g VS_{added}·d), t (d) is the digestion time and γ (d) is the lag time.

Error (%), Regression coefficient (R), determination coefficient (R^2) and standard error of estimate (σ_{est}) were calculated to evaluate the goodness-of-fit and the accuracy of the results. Error was defined as the percentage difference between the experimental and the predicted or theoretical methane yield coefficient. The kinetic parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 11).

2.8. Statistical significance tests

For this study the Analysis of variance (ANOVA) test was used in order to determine whether the different pre-treatments showed any significant variation in any of the determined parameters. A significance level (p) of 0.05 was used. PEC coefficient was computed to measure the linear association between methane and the analyzed variables. PCA was applied to the whole set of standardized variables, including methane, in order to explain the correlation structure and clarify the effect of each SHP on methane production. The PCA technique has been chosen for its reduced dimensionality ability, increasing its interpretability and therefore minimizing information loss (Jollife and Cadima, 2016). The statistical analysis was performed with the statistical programming language R (R Core Team, 2019).

3. Results and discussion

3.1. Effects of the different pre-treatments on organic matter

Figure 1 shows the total and sCOD of the substrate after each pre-treatment. No significant differences were found in tCOD between treated substrates and untreated OMSW. The untreated OMSW had a tCOD of 324±13 g O_2/kg and there were slight differences after each treatment, ranging from 323±9 g O_2/kg to 344±9 g O_2/kg. However, taking the sCOD into consideration, it can be stated that SHP at 121 ºC and 133 ºC had a positive effect on organic soluble material release as significant differences were

found. According to these results, experiment A3 showed the lowest release of organic matter (sCOD: 3978±11 mg O_2/L); experiments A1, A2, presented a similar sCOD with no significant differences (4009±11 and 4027±21 mg O_2/L, respectively). sCOD in B2 pre-treatment was 4561±24 mg O_2/L and 4585±15 mg O_2/L for B3 pre-treatment, which is slightly higher than what was observed in experiment A3. However, experiment B1, the more extreme pre-treatment, showed a significant increase in sCOD (4937±26 mg O_2/L), which was 36% higher than that observed in the untreated OMSW (3109±33 mg O_2/L).

These results are consistent with TOC, TC results (data not shown) and previous studies in which pre-treatments increased the break-down of lignocellulosic biomass (Kassaye et al., 2017).

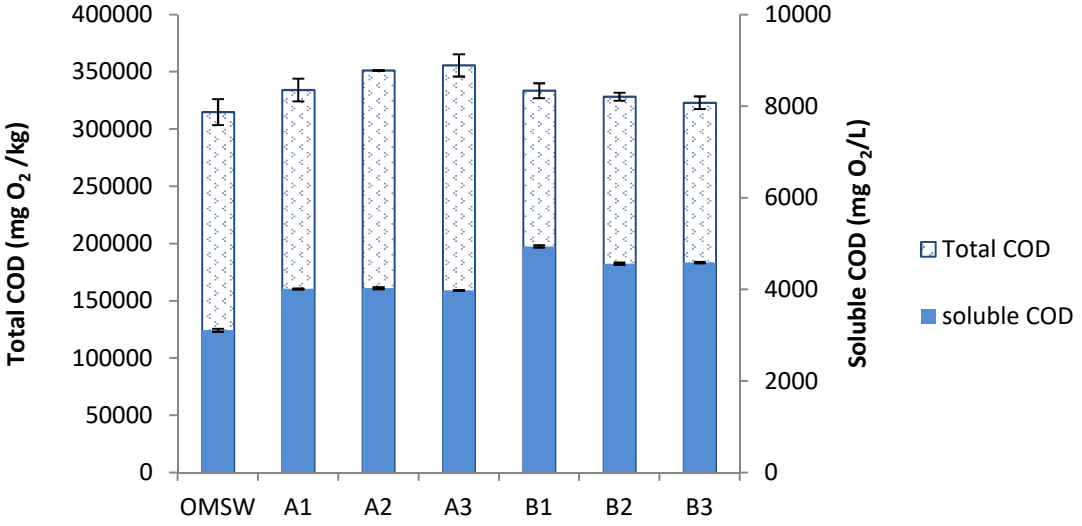

Figure 1. Chemical Oxygen Demand (COD) and Soluble Chemical Oxygen Demand (sCOD) values for untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

3.2. Effects of the different pre-treatments on lipids

The experimental results showed that pre-treatments had no effect on lipids. The total lipid concentration for untreated OMSW was 13%, and similar results with no significant differences appeared in every substrate after pre-treatment (Table 1). The lipid fraction content of diglycerides, triglycerides and free fatty acids were also constant in all cases, since the hydrolysis of olive oil occurs at temperatures higher than 180 °C (Vecchio et al., 2008).

3.3. Effect of the different pre-treatments on fiber and soluble sugars

OMSW is a lignocellulosic substrate mainly composed of three types of polymers: cellulose, hemicellulose and lignin.When the OMSW was subjected to different pre-treatments, these fibers were affected by being shortened approximately by half, both in length and width, which favoured the AD due to the fact that fibers were converted to microfibers that had a greater accessibility to bacteria than in the OMSW without pre-treatment. No pre-treatment showed significant differences between fiber length or diameter, but there were significant differences between the untreated OMSW and the different pre-treatments. It could also be observed that these treatments increased the ratio of macrofibrils, which would indicate a greater surface fibrillation (Table 2).

Table 1. Total lipids, triglycerides, diglycerides and free fatty acid contents of untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A 121 ºC and 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively) and pre-treatment B was performed at133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

Substrate	Triglycerides (%)	Diglycerides (%)	Free fatty acids (%)	Total lipids (%)
OMSW	79.5	3.0	17.5	13.2 ± 0.4
A1	79.9	3.1	17.0	12.7 ± 0.2
A2	78.5	2.7	18.8	13.2 ± 0.4
A3	79.0	2.9	18.0	12.2 ± 1.1
B1	79.2	3.0	18.5	12.6 ± 0.5
B2	79.0	3.2	17.7	12.4 ± 1.2
B3	79.9	2.9	18.0	13.2 ± 0.1

Certain degree of polymerization was observed, which increased with pre-treatment temperature and time exposure (Table 2). Sannigrahi et al. (2011) found similar results when observing that biomass from monomeric sugar could further react and form pseudo-lignin. This result was found after an acid treatment. Several authors stated that hydrothermal pre-treatment (temperature and pressure) acts as an acid catalyst (Garrote et al., 1999).

The shortened fibers of the OMSW during pre-treatment did not release more monosaccharides into the soluble phase of OMSW (Table 3).

The soluble monosaccharide study (Table 3) revealed that there were no significant differences between treated and untreated OMSW for fucose, arabinose, xylose, mannose, galactose and glucose, as can be seen in Table 3. However, in the B1 experiment, rhamnose was found to be present at 47% higher than in the other treated or untreated OMSW. This could be explained by the polymerization of released sugars which could bond with other sugars or phenols (Sannigrahi et al., 2011). In view of these results two factors must be considered regarding released sugars: on the one hand, a higher temperature and time must release more soluble sugars; while at the same time polymerization must increase with a raise in temperature and time. In this study only the rhamnose from experiment B1 (higher temperature and time) showed a net increase compared to the untreated OMSW.

Table 2. Fiber analysis for untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

Substrates	Length (mm)	Diameter (µm)	Ratio (%)	Fine Elements (%)	Viscosity (cc/g)
OMSW	0.325 ± 0.016	49.5 ± 7.4	3.583 ± 0.490	99.5 ± 0.2	44 ± 2
A1	0.135 ± 0.004	23.1 ± 1.1	3.467 ± 0.432	83.2 ± 8.7	35 ± 3
A2	0.139 ± 0.009	22.6 ± 0.1	3.760 ± 0.594	72.1 ± 8.0	34 ± 2
A3	0.144 ± 0.011	23.2 ± 0.7	4.185 ± 0.656	63.1 ± 10.7	35 ± 3
B1	0.139 ± 0.005	24.3 ± 1.5	4.338 ± 1.150	88.0 ± 5.2	33 ± 2
B2	0.143 ± 0.002	23.3 ± 1.2	3.929 ± 0.214	81.3 ± 5.9	30 ± 5
B3	0.136 ± 0.002	24.1 ± 1.3	3.946 ± 0.462	85.4 ± 6.7	34 ± 1

Table 3. Concentration of monosaccharide (μg/ml) in the soluble phase of untreated olive mill solid waste (OMSW) and in the pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

Substrate	Arabinose	Fucose	Arabinose	Xilose	Manose	Glucose	Galactose
OMSW	76.3±4.7	3.8±0.1	295.4±8.8	155.5±1.6	5304±93	15777±78	287.1±9.3
A1	81.5±9.0	0.2±0.1	291.6±10.3	132.4±7.6	5377±430	16303±189	240.5±5.2
A2	80.0±6.8	0.5±0.7	289.9±3.0	152.5±7.7	5468±24	16186±222	283.6±13
A3	72.4±0.7	0.9±1.2	278.3±13.4	129.7±1.9	5275±173	16368±116	257.5±22
B1	115±1.0	2.9±0.6	316.3±4.5	142.4±16.5	5924±74	16967±150	271.7±7
B2	91.0±16.1	2.1±0.3	300.8±16.3	154.6±12.2	5706±273	16448±701	286.7±18
B3	89.9±12.4	1.3±1.9	318.6±24.4	155.3±13.5	5723±353	16861±620	286.4±19

Nevertheless, polysaccharides (Figure 2) showed a significant increase after pre-treatment, with A1 (lower temperature and higher time) and B2 (higher temperature and intermediate time) showing the highest contents. Polysaccharides, which contain mannose, were only solubilized during the A1 pre-treatment, although the instability of mannose in an analysis method could be the reason for this absence (De Ruiter and Burns, 1987). Galactose and fucose polysaccharides were solubilized in each treatment, with A1 being the treatment that more polysaccharides with galactose and fucose solubilized. The B2 treatment showed a similar solubilization of fucose polysaccharides.

Slight solubilization of rhamnose polysaccharides were shown after every treatment. Arabinose and xylose polysaccharides were solubilized in every case, but the maximum solubilization of this polysaccharide was found during the pre-treatments A1 and B2 (Figure 2). Glucose polysaccharide was solubilized in all pre-treatments except in A3 and B1.

The presence of polysaccharides from non-glucose-monomers suggested that the fibers that were primarily affected by pre-treatments were hemicellulose, lignin and pectin (Barakat et al., 2012). Several studies showed that the critical temperature for hemicellulose degradation is within a range of 150-300 ºC, for

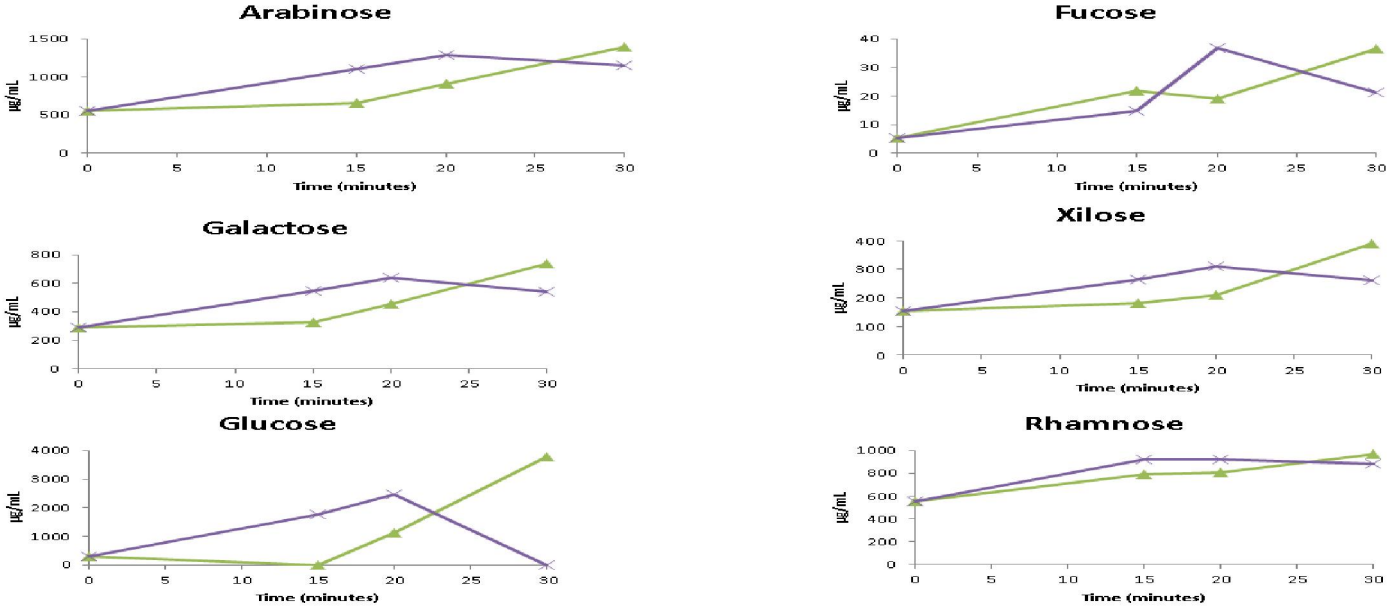

Figure 2. Variation in polysaccharides concentrations for untreated olive mill solid waste (OMSW) (time = 0) and pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A (—▲—) was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B (—x—) was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

lignin it is 150-700 ºC and that cellulose decomposed in a range of 300-400 ºC (Christoforou and Fokaides, 2016). The results from this study suggest that SHP could degrade fiber, except cellulose, at lower temperatures due to the use of pressure. However, these temperatures and pressures were not drastic enough to generate lignocellulosic-derivate by-products such as furans (Zhang et al., 2014).

3.4. Effect of the different pre-treatments on soluble phenols (SP)

The effect of pre-treatment conditions on soluble phenols is illustrated in Table 4. In all cases an increase in phenol concentration in the soluble phase was observed compared to the untreated OMSW. Therefore, SHPs can be considered as efficient extraction procedures of phenols with high antioxidant capacity. While the untreated OMSW had a total concentration of 7.91 ±0.04 g/kg, the A1 pre-treatment solubilized up to 22.9% more. The same trend was observed when comparing the temperatures of the pre-treatments. In addition, it was determined that the higher the exposure time, the higher the phenol solubilization. The maximum phenols solubilization seems to be attained at 30 minutes and at a temperature of 121 ºC (A1 pre-treatment). At 133 ºC there was a decrease in total phenol contents (9.0 ± 0.2, 8.7 ± 0.2 and 8.5±0.3 g/kg for B1, B2 and B3, respectively).

Table 4. Concentration of the main phenols (mg/kg) in the untreated olive mill solid waste (OMSW) and in the pre-treated OMSW after six different pre-treatments: A1, A2, A3, B1, B2 and B3. Pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively). DHPG: 3,4-dihydroxyphenylglycol, HT: hydroxytyrosol, T: tyrosol, Glu-HT: hydroxytytosol-glucoside, V: vanillin and Va: vanillic acid.

Substrate	DHPG	Glu-HT	HT	T	V	Va
OMSW	22.9 ± 0.3	126.9 ± 0.2	40.6 ± 0.0	142.2 ± 0.3	122.7 ± 0.2	9.35 ± 0.0
A1	405.4 ± 0.4	295.9 ± 0.2	582.3 ± 0.6	198.2 ± 0.2	296.1 ± 0.3	17.9 ± 0.0
A2	371.3 ± 0.2	275.1 ± 0.4	588.9 ± 0.5	182.2 ± 0.3	288.7 ± 0.1	32.6 ± 0.1
A3	363.9 ± 0.3	250.9 ± 0.4	409.3 ± 0.7	153.2 ± 0.1	231.3 ± 0.6	28.2 ± 0.0
B1	346.9 ± 0.2	267.7 ± 0.5	522.2 ± 0.5	208.8 ± 0.1	210.0 ± 0.2	7.3 ± 0.1
B2	348.0 ± 0.5	289.9 ± 0.2	587.2 ± 0.4	214.5 ± 0.2	259.6 ± 0.1	8.3 ± 0.0
B3	348.9 ± 0.6	300.1 ± 0.1	658.9 ± 0.8	211.4 ± 0.1	244.2 ± 0.3	14.2 ± 0.1

Abdessalem et al. (2017) reported that the percentage of phenols in dates decreased with severity of treatment because the pre-treatment solubilized a greater portion of the cell wall material, mainly hemicelluloses that can be linked with simple phenols. Temperature rather than exposure time seemed more important for phenol solubilization. It is worth mentioning that all the pre-treatments had a significant effect on total phenol release but in this work, there were no significant differences among the three A pre-treatments. On the other hand, in the B pre-treatments there were significant differences between the time exposure of 15 minutes (8.56 ± 0.08 g/kg) and the other pre-treatment durations (8.90 ± 0.25 g/kg).

The composition of individual phenols in the soluble phase of OMSW was similar to the composition previously reported by Rubio-Senent et al. (2013). The main phenols present in the soluble phase of the OMSW were 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol (HT), tyrosol (T), hydroxytytosol-glucoside (Glu-HT), vanillin (V) and vanillic acid (Va).

The initial content of DHPG in the OMSW was 22.9 ± 0.3 mg/kg. At 121 ºC the content of this phenol in the soluble phase increased, reaching values of 405.4 ± 0.4, 371.0 ± 0.2, 363.9 ± 0.3 mg/kg for pre-treatments A1, A2 and A3, respectively. At this temperature, the solubilization of this phenol increased with the pre-treatment duration. An increase in the pre-treatment temperature led to lower DHPG solubilization, probably as a result

of phenol degradation (Umamaheswari and Rajaram, 2014) or the absorption of simple phenols to a polymeric phenolic fraction which was enhanced by the severity of the thermal pre-treatment (Rubio-Senent et al., 2012). Therefore, the DHPG varied from 346.9 ± 0.2 mg/kg for pre-treatments B1, to 348.0 ± 0.5 and 348.9 ± 0.6 mg/kg for B2 and B3, respectively. Temperature, pressure and time affected DHPG solubilization. The statistical analysis revealed that DHPG contents in the soluble phase were significantly different for each case. At 121 ºC (A experiment) this phenol content increased with time, while at 133 ºC the phenol content decreased slightly with time.

Temperature and pressure had a great effect on Glu-HT solubilization. The content of this phenol in the soluble phase of OMSW was 126.9 ± 0.2 mg/kg. During the pre-treatment at 121 ºC (1.1 bar) the concentration of Glu-HT reached 295.9 ± 0.2 mg/kg after 30 minutes, 275.1 ± 0.4 mg/kg after 20 minutes and 250.9 ± 0.4 mg/kg after 15 minutes. A more severe pre-treatment (133 ºC, 2.1 bar) solubilized 267.5 ± 0.5 mg/kg after 30 minutes, 289.9 ± 0.2 mg/kg after 20 minutes and 300.1 ± 0.1 mg/kg after 15 minutes. Like DHPG, the Glu-HT concentration in the soluble phase increased with time at 121 ºC; while it decreased at 131 ºC, similar results were obtained by Abdessalem et al. (2017).

In the case of HT, the lowest value in the soluble phase was obtained for the untreated OMSW (40.6 ± 0.0 mg/kg), while the highest value was observed for B3 pre-treatment (658.9 ± 0.8

mg/kg). However, when the time of exposure increased the concentration of HT in the soluble phase decreased drastically up to 522.2 ± 0.5 mg/kg after 30 minutes. When the samples were subjected to 121 ºC the maximum solubilization of this phenol was produced after 20 minutes (588.9 ± 0.5 mg/kg); while after 30 minutes it was 582.3 ± 0.6 mg/kg, and the minimum solubilization was produced after 15 minutes (409.3 ± 0.7 mg/kg).

Table 4 shows the release of T after each pre-treatment. The initial concentration of T in the soluble phase of the OMSW was 142.2 ± 0.3 mg/kg. T looked like a more thermostable phenol and had its maximum concentration at 133 ºC after 20 minutes, reaching up to 214.5 ± 0.2 mg/kg. At 121 ºC the maximum solubilization was achieved after 30 minutes although the concentration was lower than at 133 ºC.

The initial concentration of Va in the soluble phase of the untreated OMSW was 9.3 ± 0.2 mg/kg. At 133 ºC the concentration decreased to 7.3 ± 0.1 mg/kg and 8.3 ± 0.1 mg/kg after 30 (B1) and 20 (B2) minutes, respectively; while after 15 minutes (B3) the concentration was higher (14.2 ± 0.1 mg/kg). Nevertheless, at 121 ºC the Va concentration increased regardless of the time, reaching its maximum concentration after 20 minutes of pre-treatment exposure (32.6 ± 0.1 mg/kg). These results suggest that at 121 ºC the solubility of Va was faster and greater than its degradation; while at 133 ºC after 20 minutes, the degradation of Va occurred faster than its solubilization.

Although the concentration of V increased with both pre-treatments, during A pre-treatment the concentration of this phenol was higher when the time of exposure increased. The most severe pre-treatment steadily increased the concentration of V in the soluble phase but when the time of exposure was 30 minutes, the concentration of this phenol decreased in the soluble phase.

By comparing the data on individual phenol content (Table 4) with the experimented methane production values (Figure 3) threshold concentrations of T and HT could be established after which the AD process may be inhibited. Specifically, HT and T concentrations equal to or lower than 582 mg/kg and 198 mg/kg cannot be considered as inhibitors of the AD process because these concentrations did not significantly affect methane production.

The most remarkable data was that there was no presence of degradation products such as hydroxymethylfurfural or furfural, which are among the main inhibitors for AD (Monlau et al., 2014), although furfural could have been lost because of its volatility (Bolado-Rodríguez et al., 2016).

3.5. Effect of SHP on AD rate and methane yield

Figure 3 shows the methane production of different pre-treated OMSWs and untreated OMSW over a period of 30 days. The methane yield obtained during AD of 100% OMSW was 341 ± 22 mL CH_4/g VS added. This value of methane yield obtained for the BMP test of OMSW was in accordance with previous studies

(Rincón et al., 2013). After 30 days of experiment, the maximum methane yield obtained was 383 ± 2 mL CH_4/g VS_{added} for A1 pre-treatment. The A2 pre-treatment obtained 352 ± 8 mL CH_4/g VS_{added}, and finally, A3 produced 315 ± 10 mL CH_4/g VS_{added}. By contrast, B pre-treatments reached values of 308±39, 290±16 and 274±6 mL CH_4/g VS_{added} for B1, B2 and B3, respectively. Therefore, only the pre-treatments A1 and A2 exhibited higher methane yields compared to the AD of untreated OMSW. Thus, the experimental methane yield improvement was 36% and 35% for A1 and A2, respectively.

Many authors have pointed out a high increase in methane yield as one of the benefits of the pre-treatments, but sometimes the solubilization of organic matter is not so good for the ultimate methane yield (Lizasoain et al., 2016; Razavi et al., 2019). For instance, in this study the pre-treatment which solubilized more organic matter was B1 (133 ºC, 2.1 bar, 30 min), but it showed a methane yield which was lower than that obtained for untreated biomass. By contrast, A1, which only increased the organic matter solubilization by 10%, showed a markedly higher methane yield compared to the untreated substrate.

a)

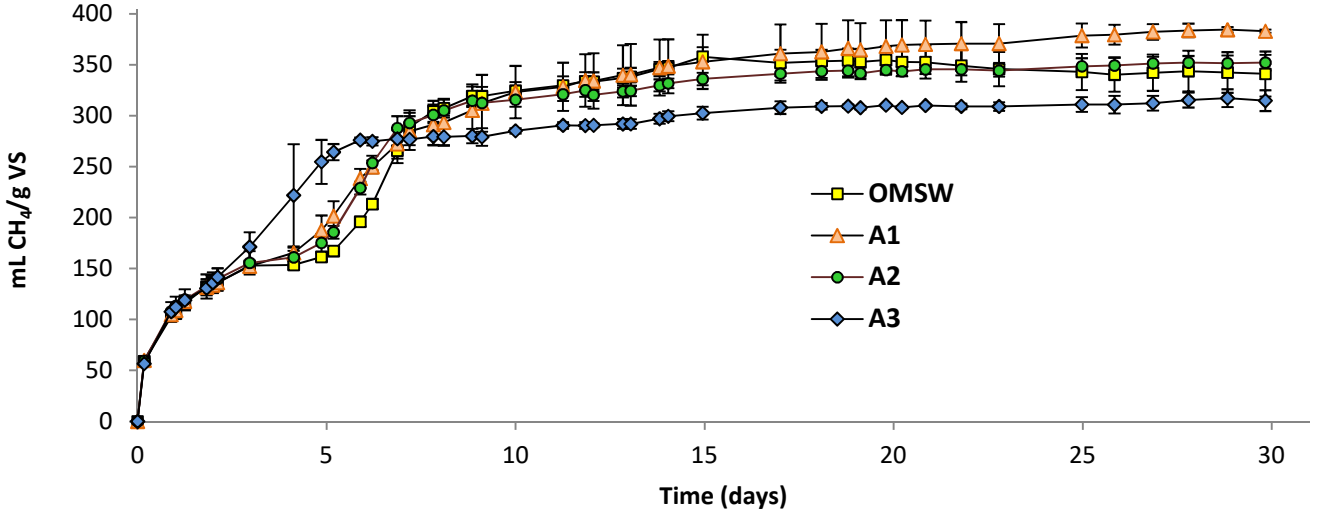

Figure 3a. Cumulative methane yield obtained from untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: pre-treatment A (Figure (a)) was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively).

b)

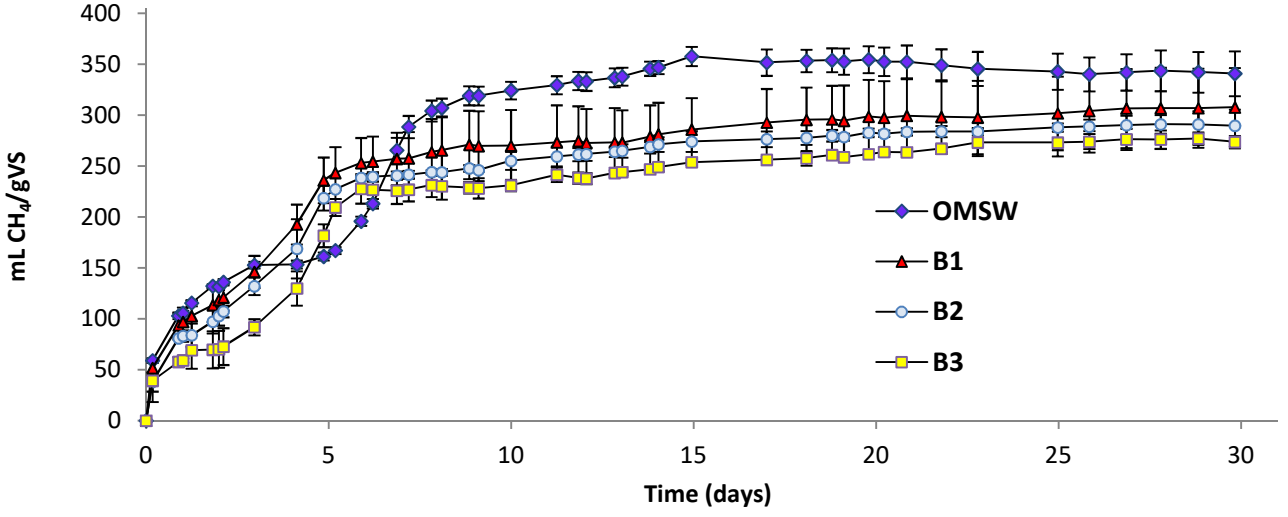

Figure 3b. Cumulative methane yield obtained from untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: pre-treatment B (Figure (b)) was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively).

On the other hand, B pre-treatment modified the kinetics of OMSW degradation by removing part of the lag period, but finally, the methane yield was 45% less than that obtained from the untreated OMSW (Barakat et al., 2012).

Exposure to high temperature and high pressure during hydrothermal pre-treatment could therefore account for a significantly lower polysaccharide and phenol solubilization due to the fact that both are degraded in other molecules, although furfural o 5-HMF was not found in this case.

3.6. PEC and PCA analysis

In order to link up the effect of pre-treatment and the methane production, a PEC was carried out. A positive correlation was observed between methane and the Va ($r = 0.343$) and a negative correlation with sCOD ($r = -0.519$) and with the T ($r = -0.340$). The other phenols (HT, Glu-HT, V and DHPG), soluble polysaccharides and the fiber size did not demonstrate a high correlation with methane production.

PCA analysis of the biochemical composition of the substrates after SHPs shows that nearly 95% of variability could be explained by the first three principal components (Figure 4). The first principal component (PC1) expressed 66.81% of the overall variance. The second (PC2) and the third (PC3) principal component expressed 14.64 and 12.83%, respectively. All parameters (phenols, polysaccharides and sCOD) except methane

were positioned close to PC1. Instead, they were opposed to fiber length and diameter. PC2 were close to the Va. The correlated polysaccharides (arabinose, xilose, galactose and glucose) and fiber length and diameter were also clustered in the direction of PC2 but with negative coordinates. PC3 was positive connected with sCOD and negative with Va, glucose and methane (Figure 4).

A1 and A2 samples that reached the highest methane yield (380±5 and 350±6 ml CH_4/ g VS_{added}, respectively) were gathered together and can be explained by the polysaccharides of content in glucose, arabinose, xylose, galactose and the Va. B pre-treatments, the lowest methane yield, were linked with sCOD and the T.

3.7. Effect of SHP on process kinetics

Table 5 shows the main performance and kinetic parameters obtained from the application of the TF model to the experimental data of methane production-time corresponding to the different BMPs or tests carried out. As can be seen, the high R and R^2 values as well as the low values of errors and standard errors of estimates (σ_{est}) indicated that the experimental data correctly fit the proposed model.

a)

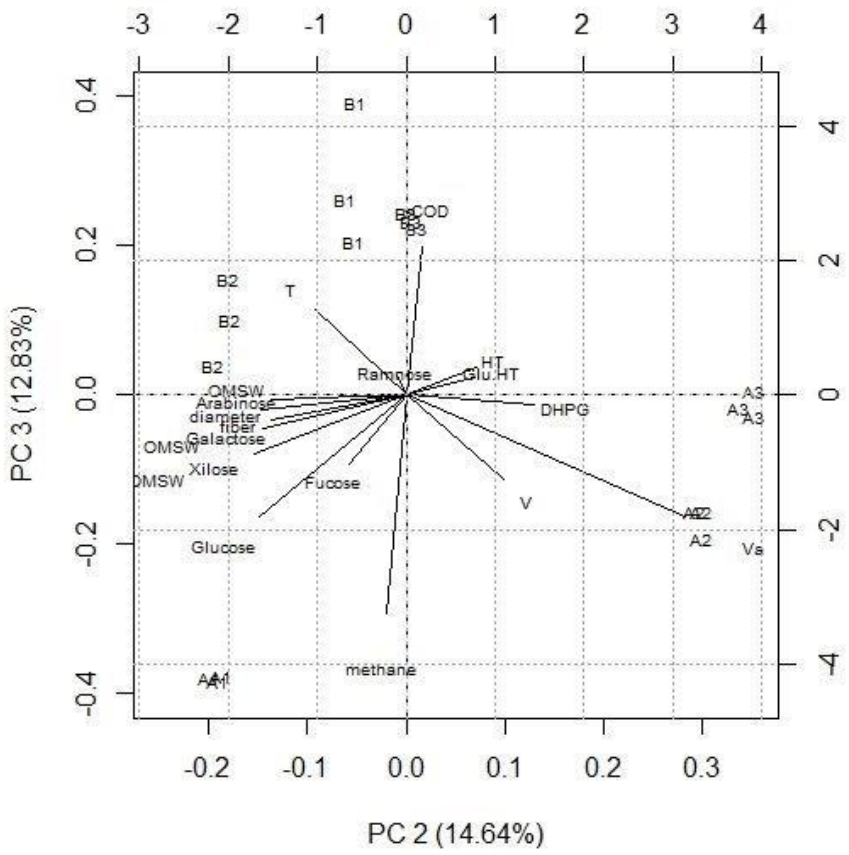

Figure 4a. Principal component analysis of the biochemical composition of the untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively). PC2 and PC3: principal component 2 and 3, respectively.

b)

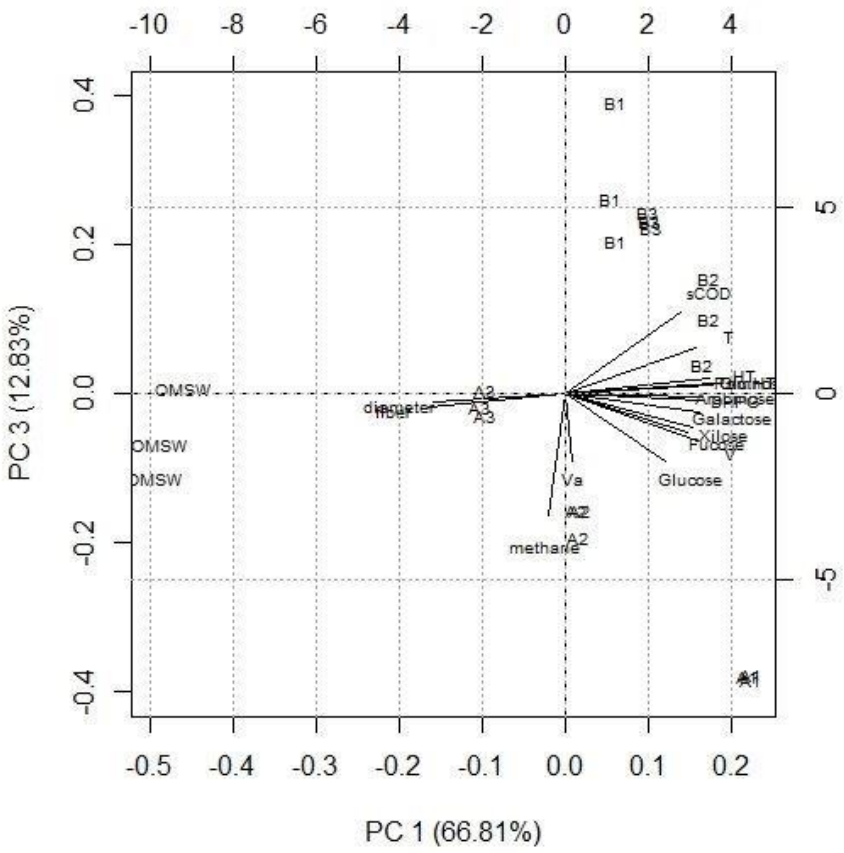

Figure 4b. Principal component analysis of the biochemical composition of the untreated olive mill solid waste (OMSW) and pre-treated OMSW after six different pre-treatments: pre-treatment A was carried out at 121 ºC and pressure of 1.1 bar for 30, 20 and 15 min (A1, A2 and A3, respectively), pre-treatment B was performed at 133 ºC and 2.1 bar for 30, 20 and 15 min (B1, B2 and B3, respectively). PC1 and PC3: principal component 1 and 3, respectively.

As can be seen in Table 5, for the thermal pre-treatment carried out at 1.1 bar pressure and 121 ºC temperature (experimental serie A), the theoretical ultimate methane yield increased from 306 ± 3 mL CH_4/g VS (A3) to 380 ± 5 mL CH_4/g VS (A1) when the exposure time during pre-treatment augmented from 15 to 30 minutes. This represents a 24.2% increase when the operation time increased between the above-mentioned values. This increase was statistically significant with a probability level of 95%. In the same way, for the experiments performed at 2.1 bar pressure and 133 ºC (experimental serie B), the predicted ultimate methane yield also increased significantly from 270 ± 4 mL CH_4/g VS (B3) to 296 ± 3 mL CH_4/g VS (B1) when the operation time during pre-treatment increased from 15 to 30 minutes. Therefore, an increase of only 9.6% was appreciated in this case. Moreover, in the experimental series B, all predicted methane yield values were lower than the ultimate methane yields obtained during the experimental series A and also lower than that obtained for untreated OMSW. The same trend was observed with the experimental values for methane yield.

Table 5. Kinetic parameters obtained from the Transference Function model applied to the different Biochemical Methane Potential (BMP) assays. Where B_{max}: the ultimate methane production, R_{max}: the maximum methane production rate γ: the lag time, R: regression coefficient, R^2: determination coefficient and σ_{est}: standard error of estimate. OMSW: untreated olive mill solid waste, A1, A2 and A3: pre-treated OMSW under conditions A1, A2 and A3 (121 ºC and 1.1 bar for 30, 20 and 15 min, respectively) and B1, B2 and B3: pre-treated OMSW under conditions B1, B2 and B3 (133 ºC and 2.1 bar for 30, 20 and 15 min, respectively).

Substrate	B_{max} **(mL CH_4/g VS)**	**Error (%)**	R_{max} **mLCH_4/(g VS·d)**	γ**(d)**	**R**	R^2	σ_{est}*
OMSW	358 ± 7	5.0	70 ± 5	0	0.969	0.941	25.6
A1	380 ± 5	0.8	69 ± 3	0	0.986	0.973	18.0
A2	350 ± 6	0.6	73 ± 5	0	0.979	0.956	20.9
A3	306 ± 3	2.9	102 ± 5	0	0.989	0.979	12.0
B1	296 ± 3	3.8	84 ± 4	0	0.988	0.977	12.5
B2	283 ± 3	2.4	73 ± 3	0	0.991	0.983	10.6
B3	270 ± 4	1.4	58 ± 4	0	0.983	0.967	15.0

Among the different experimental conditions tested, only the experiment A1 gave either the predicted and experimental ultimate methane yields higher than that obtained for untreated OMSW. Therefore, the operational conditions for pre-treatment A1 allowed for obtaining a substrate with an anaerobic biodegradability higher than that obtained from the untreated OMSW. This resulted in an increase of 6.1% in the methane yield of the pre-treated OMSW at the above conditions (A1) compared to untreated OMSW. This fact may be attributed to the considerable reduction in complex and inhibitor compounds, i.e. phenolic compounds, present in the OMSW pre-treated at these conditions (A1) compared to untreated OMSW. Momayez et al. (2018) described an enhancement of up to 26% in methane production in the AD from thermally pre-treated rice straw (190 ºC, 30 minutes)

Potent AD inhibitors can be formed after thermally pre-treated lignocellulosic biomass (Ghasimi et al., 2016; Paul and Dutta, 2018). Sometimes the solubilization of organic matter through thermal pre-treatment is not so good for the ultimate methane yield (Lizasoain et al., 2016; Razavi et al., 2019). In fact, the methane yields obtained in the BMP tests of untreated and autoclaved food waste were 0.501 and 0.445 m^3 CH_4/kg VS, respectively, which were probably due to the formation of refractory compounds such as melanoidins, that can affect biodegradability and, consequently, methane production (Pagliaccia et al., 2016).

The calculated lag times (λ) were found to be zero in all cases, because the easy and most available biodegradable components of all substrates were quickly consumed in all the AD processes studied (Li et al., 2012).

The R_{max} values presented a somewhat different trend to that observed for B_{max} in the different experiments carried out. The highest R_{max} value was found for the experiment A3 with 102 ± 5 mL CH_4/g VS·d. This value was 45.7% higher compared to that obtained for untreated OMSW (70 ± 5 mL CH_4/g VS·d). It has been recently reported that the performance of thermal pre-treatment is influenced by both temperature and exposure time (Jain et al., 2015) and the optimal temperature depends on the substrate characteristics. On the contrary, the slowest biomethanization process took place for the B3 conditions. This decrease in R_{max} for the pre-treatment carried out at higher temperature and pressure conditions is a good indication that compounds in this pre-treated fraction might have a lower initial availability for its AD (Paul and Dutta, 2018). In addition, a higher temperature in the pre-treatment could derive from the degradation of some complex phenolic compounds to undesirable compounds such as furfural and 5-HMF, which have been considered as inhibitory for AD processes (Ghasimi et al., 2016; Paul and Dutta, 2018).

4. Conclusions

The SHP A1 (121 ºC, 1.1 bar for 30 minutes of exposure time) increased the methane yield of the pre-treated OMSW by 36% compared to the value obtained for untreated OMSW. However, the A1 pre-treatment did not generate the maximum solubilization of the waste, which was achieved in the B3 pre-treatment (133 ºC, 2.1 bar for 15 minutes). The SHPs helped to break the OMSW fiber in half both in length and in diameter, helping to solubilize sugars in the form of polysaccharides. The pre-treatments also helped to solubilize phenolic compounds achieving high concentrations of valuable compounds such as HT, 658.9±0.8 mg/kg, and DHPG, 405.4±0.4 mg/kg, moreover, some of them being beneficial for the AD process at the concentration ranges tested (7.3 mg/kg for vanillic acid). However, it was found that T concentrations higher than 198 mg/kg were inhibitory for the AD process, bringing about a decrease in methane production. The TF model was demonstrated to be a proper tool for evaluating the performance and kinetic parameters of the AD of thermally-pre-treated OMSW. The A1 thermal pre-treatment conditions allowed for increasing the predicted methane yield by 6.1% compared to untreated OMSW. The highest value for maximum methane production rate, R_{max}, was obtained at the above-mentioned conditions but at 15 min of exposure time (A3).

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Chapter 2

The influence of microalgae addition as co-substrate in anaerobic digestion processes

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Abstract

Growth microalgae could be used as co-substrates in anaerobic digestion processes to produce biogas of a high calorific value, which could be expended as heat or electricity in cogeneration engines. Lignocellulosic and high carbon content wastes, due to their characteristics, hinder anaerobic digestion processes. The use of microalgae as a co-substrate with high carbon-content residues can adjust the C/N ratio and thereby obtain, in some cases, a higher biogas production and greater biodegradability of wastes during anaerobic digestion than without co-digestion options. In addition, microalgae and cyanobacteria are photosynthetic microorganisms that can produce oxygen and oxidize the organic matter and NH_4^+ contained in wastewaters. The growth of microalgae in industrial effluents and wastewaters can considerably reduce the organic matter contained in them and their pollutant load. This growth can take advantage of the nutrients that still remain in industrial effluents, avoiding the use of clean water for the growth of biomass. The chapter will focus on an overview of microalgae anaerobic co-digestion with different wastes and the benefits of this option.

1. Introduction

One of the main challenges that society will face in the near future is the potential lack of traditional energy sources. The rising price of fossil based fuels and their negative environmental impact combined with increasing energy consumption make the demand for renewable energy sources greater. For this reason, a wide variety of biomass has been investigated in order to evaluate its potential as a proper feedstock for the production of different biofuels such as biodiesel, bio-methanol, bio-hydrogen, bio-oil and biogas (Santos-Ballardo et al., 2016). Nevertheless, the increasing world population will need an adequate food supply which could be a problem if cultivated land is destined to biofuels and not to human or animal feed. Thus, non-edible biomass which does not require usable land would be a promising alternative. In this regard, the attention of the scientific community has been focused on oleaginous microorganisms like microalgae and cyanobacteria in recent years. Microalgae do not need agricultural land for growing, they improve air quality through CO_2 removal, and they require minimal use of fresh water resources (Najafi et al., 2009).

The main properties that make some microalgae and cyanobacteria good alternatives as biomass for biofuel production include: their highly efficient photosynthetic mechanisms (MacIntyre et al., 2002); their elevated biomass production of up to 5-10% vs. 0.5-3% in plants (Laws et al., 1988); their growth rates,

which are 5-10 times faster than land-based feedstock (Geider, 1987); and their accumulation of lipids and carbohydrates (Jankowska et al., 2017; Santos-Ballardo et al., 2016). However, the main nutrients required for the growth of microalgae and cyanobacteria are inorganic carbon (some microalgae species are able to utilize organic carbon), inorganic nitrogen (ammonium or nitrate) and phosphorous. These requirements can make their growth expensive in some cases. For example, to generate 1 kg of biodiesel in fresh water requires 3.726 kg of water, 0.33 kg of nitrogen and 0.71 kg of phosphate (Yang et al., 2011). However, it is now known that microalgae can be grown using nutrient-rich wastewaters like digestates from anaerobic digestion processes such as liquid supernatants rich in nitrogen and phosphorous, animal manure or textile wastewater (Huy et al., 2018), food wastewater (Ji et al., 2015a), and aquaculture wastewater (Andreotti et al., 2017) among others. Even in saline waters, which are usually rich in nitrogen (Ryther et al., 1971), this disadvantage to the water quality for growth is easily overcome. In the same way, recycling harvest water reduces the water and nutrient requirements by 84 and 55%, respectively (Yang et al., 2011). The use of wastewater for microalgae and cyanobacteria growth present the advantage of reducing the cost and environmental impact of the system by reducing the use of clean water and mineral nutrients while biomass productivity is comparable to that obtained from a synthetic medium (Li et al., 2017b).

Microalgae also uptake carbon by the photosynthesis process during growth, reducing CO_2 emissions ten times more efficiently than those reduced in a forest (Jankowska et al., 2017; Thorin et al., 2017), by transforming CO_2 into new biomass. Microalgae culture can contribute simultaneously to both CO_2 fixation and wastewater treatment (Razzak et al., 2017). Hirata et al. (1996) found that a batch culture of *Chlorella* sp. UK001, using sunlight as a light source and growing at a mesophilic temperature with pH between 5.5 to 6.0 achieved a mean rate of CO_2 fixation during the culture of 0.0318 g CO_2/L·d. Maeda et al. (1995) found that another strain of *Chlorella*, strain *Chlorella* sp. T-1, was an ideal candidate for the biological fixation of CO_2 exhausted by a coal-fired thermal power plant. Other authors demonstrated that the strain *Chlorella* sp. MTF-15 could efficiently utilize CO_2, NO_x and SO_2 from the different flue gases obtained in a steel plant: flue gas from a coke oven, flue gas from a hot stove and from a power plant for cultivation (Kao et al., 2014).

Furthermore, the growth of microalgae in wastewaters aids in the treatment of pollutant wastewaters and could be introduced as a tertiary treatment (Arias et al., 2018; Brown and Shilton, 2014; Guldhe et al., 2017; Rahman et al., 2015; Udaiyappan et al., 2017). In addition, the capacity of microalgae for synthesizing and accumulating different compounds, which could be considered for pharmaceutical and nutraceutical purposes, is an added value (Olaizola, 2003). The different metabolic pathways of fresh and

marine water algae provide promising sources of fatty acids, steroids, carotenoids, polysaccharides, lectins, and halogenated compounds, among others (Sathasivam et al., 2017). Microalgae are the most promising sources of pigments and natural carotenoids of commercial interest, including β-carotene, lutein and astaxanthin (Hu et al., 2018; Pulz and Gross, 2004). Furthermore, the carotenoids produced by microalgae are devoid of the toxic effects associated with synthetic derivatives (Hu et al., 2018). Microalgae are also used as nutritional supplements for animals and humans because of the quality of the proteins that they produce. *Spirulina, Chlorella, Dunaliella* or *Nostoc* are microalgae and cyanobacteria grown for human consumption (Pulz and Gross, 2004).

The most common systems for the cultivation of microalgae used for biogas production are open ponds (OPR), photo-bioreactors (PBR) and hybrid systems. OPR are relatively low-cost systems, although, the biomass yield is lower and contamination is quite common. PBR systems permit a higher control over microalgae growth and its optimization; nevertheless, the cost of these systems is much higher than OPR (Jankowska et al., 2017).

Different approaches to microalgae as biomass for biofuel extraction have been studied, but not all of them with the same success. Regarding lipid accumulation for biodiesel production, algae grown in wastewater typically showed lipid mass fractions in the volatile suspended solids (VSS) in the range of 4.9-11.3%. This fraction is much lower than that recommended for economical

biodiesel production (Wang and Park, 2015). In order to enhance the energy potential of microalgae and cyanobacteria, anaerobic digestion has been studied (Jankowska et al., 2017; Santos-Ballardo et al., 2016) as another alternative. Anaerobic digestion is a complex biological process in which organic raw materials are converted to biogas through the action of a consortium of microorganisms that are sensitive to or completely inhibited by oxygen. Biogas is a mixture of methane (60-70%) and carbon dioxide (30-40%), and traces of other constituents (hydrogen, hydrogen sulphide, etc.) of high energetic value from 20 to 25MJ/m^3 (Borja and Rincón, 2017). Around 31 m^3 of methane per 100 kg of Chemical Oxygen Demand (COD) fed into an anaerobic reactor can be produced, with a maximum energetic value of 108 kWh as electric energy or 308 kWh as heat. It has been reported in the literature that microalgae and cyanobacteria can be potentially used for energy recovery through anaerobic digestion, although the yields obtained depend highly on the specie and the operational conditions of growth (Mussgnug et al., 2010; Santos-Ballardo et al., 2016). The initial studies in the fifties (Golueke et al., 1957) obtained values of methane yields of 0.17 to 0.32 L CH_4/g SV_{added} for *Chlorella* and *Scenedesmus* in batch processes, although other authors found higher values of methane yield: at 0.587 L CH_4/g SV_{added} and 0.505 L CH_4/g SV_{added} for *Chlamydomons reinhardtii* and *Dunaliella Salina*, respectively (Mussgnug et al., 2010).

Growth conditions could affect the morphology and intracellular substances in microalgae. The thickness of the cell walls in microalgae could be increased due to stressed growing conditions, which could be a disadvantage during anaerobic digestion (Wang and Park, 2015). In addition, microalgae and cyanobacteria present a low C/N ratio which could lead to an ammonium accumulation and result in an inhibition of the digestion process. Samson and LeDuy (1986) reported concentrations of ammonia of up to 7000 mg/L for the anaerobic digestion of the protein-rich cyanobacteria *Spirulina maxima*. However, the use of microalgae as co-substrate with other substrates or feedstocks in anaerobic co-digestion processes can improve these limitations and bring certain benefits. Anaerobic co-digestion is the simultaneous anaerobic digestion of two or more substrates and it is a proven approach to overcome the drawbacks of single digestion (Mata-Alvarez et al., 2014). Mata-Alvarez et al. (2000) in the year 2000 already wrote: “The use of a co-substrate, in most cases improves biogas yield due to positive synergisms established in the digestion medium and the supply of missing nutrients by the co-substrates”. Co-digestion has several advantages: adjusting the C/N ratio, improving nutrients and diluting inhibitor compounds (Hartmann and Ahring, 2005). The co-digestion of microalgae with high carbon biomass leads to a better balanced substrate for anaerobic digestion (Li et al., 2017a; Thorin et al., 2017; Wang and Park, 2015). Nevertheless, there are some problems that must be solved, such as the breakage of the

thick cellular walls in some microalgae and cyanobacteria. Prospective methods could be different kinds of pre-treatments before anaerobic digestion in some particular cases.

Nonetheless, due to the high variety of microalgae and cyanobacteria and the wide range of different uses, it is not clear yet what the most effective process for bio-fuel production is. Although to this respect, some authors suggest that the direct use of microalgae or cyanobacteria in an anaerobic co-digestion process is the best choice, while other researchers propose that the best choice is to produce biofuel as a first step followed by an anaerobic digestion of the residual by-products (Santos-Ballardo et al., 2016). This chapter aims at providing a current perspective of microalgae exploitation as biomass in anaerobic digestion and co-digestion processes and to show the advantages of their growth in wastewater as well as their growth in anaerobic digestates.

2. Microalgae growth in wastewaters

The cultivation of microalgae in wastewater has long been recognized as a viable option for sustainable biomass production and wastewater treatment (Brown and Shilton, 2014; Guldhe et al., 2017; Rahman et al., 2015; Udaiyappan et al., 2017). The main nutritional requirements for microalgae growth include nitrogen, phosphorus, and micronutrients such as iron, magnesium, and calcium which are present in wastewater. Recent developments in microalgal research have demonstrated that microalgae have the

required metabolic potential to effectively reduce high concentrations of nutrients such as carbon, phosphorous and nitrogen present in different wastewater streams (Guldhe et al., 2017). Some species of microalgae have the ability to take up other pollutants, such as heavy metals and harmful chemicals (Udaiyappan et al., 2017). Therefore, microalgae can be used to serve a dual purpose for the treatment of wastewater as well as generating biomass for various applications because microalgae are rich in carbohydrates, proteins and lipids.

Various wastewater streams including municipal, industrial, agricultural wastewater as well as primary and secondary effluent, centrate and anaerobic digestion effluent were exploited as suitable nutrient media for microalgae cultivation. Each wastewater stream has its own characteristics and challenges such as nutrient variability and the presence of potential inhibitors that could impact microalgal growth. Recently, many investigations have been developed to overcome challenges such as low nutrients, high turbidity, bacterial contamination and specific toxic materials associated with different wastewater.

The types of wastewater utilized for algae cultivation also affect the scope of biomass for various applications (Guldhe et al., 2017).

An alternative for recovering energy from microalgae is based on the application of anaerobic digestion processes (Torres et al., 2013). In such processes, all organic matter (proteins, carbohydrates and lipids) present in microalgae biomass would be

converted into methane and carbon dioxide (biogas). Several advantages are recognized when energy production from whole microalgae through biogas generation is considered: biogas production involves high energy yields; biogas production would not require microalgae biomass drying (it involves wet fermentation); biogas can be used to produce heat and electricity through co-generation; microalgae cultures can be used for biogas upgrading (i.e. CO_2 biosequestration), etc. However, some microalgae have a very low C/N ratio, which hinders and inhibits a further anaerobic digestion. Ammonia toxicity and recalcitrant cell walls are commonly cited causes of the low methane yields found in the anaerobic digestion of some microalgae (Fernández-Rodríguez et al., 2014). Moreover, anaerobic co-digestion of microalgae with other types of biomass such as solid and liquid wastes is quite feasible (Torres et al., 2013). The benefits of co-digestion lie in balancing the C/N ratio in the co-substrate mixture, as well as macro and micronutrients, pH, inhibitor/toxic compounds and dry matter (Fermoso et al., 2016).

The main phyla (and species) of microalgae that are being used for biogas production through anaerobic digestion and co-digestion processes are (Guldhe et al., 2017; Rashid et al., 2018; Udaiyappan et al., 2017):

-Chlorophytes, such as *Chlorella* sp./*vulgaris/sorokiniana*; *Scenedesmus* sp./*quadricauda/obliquus*; *Dunaliella salina*;

Nannochloropsis salina; *Botryococcus brauinii*; *Micractinium* sp. and *Selenastrum capricornotum*.

-Haptophytes, such as *Isochrysis galbana*.

-Cyanobacteria, such as *Arthrospira platensis* and *Oscillatoria tenuis*.

-Binary and mixed culture systems: In mixed culture systems, different microorganisms develop a synergetic relationship and live together by benefiting each other. For instance, in a binary system a photosynthetic microalga is grown with a heterotrophic microalga or bacteria. In this matrix, microalgae produce oxygen and organic compounds which are utilized by co-existing heterotrophic microorganisms.

2.1. Chlorophytes

2.1.1. *Chlorella* genus

The growth of the green algae *Chlorella* sp. in wastewater after primary settling of a local municipal wastewater treatment plant was evaluated by Wang et al. (2010). They observed a growth rate of 0.429 d^{-1} with excellent removal of ammonium (NH_4^+-N) (74.7%), P (90.6%) and COD (56.5%). These authors also investigated the growth of *Chlorella* sp. using different phases

(raw, secondary and centrate) and have demonstrated that the growth rate of microalgae and nutrient removal efficiencies were proportional to the nutrient concentration of the wastewater selected for its cultivation with the highest growth in centrate followed by raw wastewater. Osundeko and Pittman (Osundeko and Pittman, 2014) reported a high sodium concentration of 400 mg/L in sludge liquor/centrate which can be toxic to freshwater microalgal species, though some *Chlorella* sp. are tolerant to salinity. More recently, Lu et al. (2015) evaluated the biomass productivity and nutrient removal capacity of *Chlorella* sp. in raw dairy wastewater using both indoor bench-scale and outdoor pilot-scale photobioreactors. Results from this study have shown a higher biomass productivity of 260 mg/(L·d) and high nutrient (N and P) removal (83.3 and 38.3 mg/(L·d), respectively) in indoor bench-scale cultures when, compared to outdoor pilot-scale cultures with biomass of 110 mg/(L·d) and nutrient removal of 41.3 mg/(L·d) for N and 6.5 mg/(L·d) for P. These differences could have resulted due to the uncontrolled environmental and operational factors that might have affected the microalgae growth during outdoor cultivation.

Nutrient limitation is one of the key challenges for microalgal cultivation in secondary/tertiary wastewater. The supplementation of nutrients is proposed as an alternative method to overcome the nutrient limitations in wastewater. In this sense, Cabanelas et al. (Cabanelas et al., 2013) identified the potential of coupling a

wastewater treatment plant effluent with glycerol for supporting the mixotrophic production of *Chlorella vulgaris* and *B. terribillis*. The cultivation of *Chlorella vulgaris* in mixotrophic mode was also studied in a mixture of primary and secondary wastewater with different ratios (25, 50 and 75 vol percent of the primary wastewater). It was observed that using 25% of the primary wastewater and 75% secondary wastewater resulted in 100% COD removal, 100% ammonium removal and 82% nitrate removal (Ebrahimian et al., 2014).

Recently, Ansari et al. (2017) studied the cultivation of *Chlorella sorokiniama* in aquaculture wastewater with sodium nitrate supplementation and observed comparable biomass yields to the synthetic medium. In their study, they also observed high ammonia, nitrate, COD and phosphate removal and proposed that treated water can be redirected towards aquaculture. The biomass obtained in this study showed sufficient lipid, carbohydrate and protein concentrations to be used as feed supplement. Ramanna et al. (2014) supplemented 1.5 g/L urea as a cheap N source for the cultivation of *Chlorella sorokiniana* and achieved a biomass production of 0.218 g/L. A supplementation strategy can yield high biomass productivities; however, it depends on the nutrient composition of the wastewater used and the requirements of the selected microalgal strain.

For the realization of microalgal CO_2 capture and utilization, the selection of microalgal species tolerant to CO_2 from various

environments and the characterization of growth influencing environmental factors are required (Lee et al., 2015). The proper selection of species and optimized cultivation conditions i.e., light intensity, temperature, nutrient availability and pH can maximize CO_2 sequestration. *Chorella* sp. has been widely reported to possess good carbon sequestration potential. Previous studies have obtained hydrocarbons from microbial lipids for their conversion into sustainable fuels as a substitute for fossil hydrocarbons. Furthermore, microalgae have significant applications in the production of valuable materials in the food and pharmaceutical industries, resulting in a high value-added process in the bio-sequestration of CO_2 (Lee et al., 2015).

Microalgae with a lipid content of lower than 40% of their dry weight makes the anaerobic digestion route more feasible than biodiesel in terms of energy recovery. Ras et al. (2011) proposed coupling the process of microalgal biomass production and anaerobic digestion. In this process *Chlorella vulgaris* was cultivated using the nutrient rich digestate from an anaerobic digester; the microalgal biomass was then anaerobically digested to produce methane. In a later study, with hydraulic retention time (HRT) of 28 days, 51% COD removal and methane production of 240 mL/g VSS were achieved. The use of microalgae as a feedstock for bioethanol production is considered to be a sustainable approach to bioethanol production. Microalgal species such as *Chlorella* store energy in the form of starch (Ho et al.,

2013). The starch accumulated in the microalgae can be easily hydrolyzed to glucose using chemical or enzymatic methods. The sugar produced can be subsequently fermented to ethanol. Ho et al. (2013) investigated the potential of *C. vulgaris* PS-E as the bioethanol feedstock. This species contains 51% carbohydrates which were hydrolyzed through an enzymatic process to give a glucose yield of 0.461 g glucose/g dry biomass. The ethanol yield obtained in their study was 11.7 g/L.

Chlorella vulgaris was also reported to be a successful bioremediation agent of palm oil mill effluent (POME), with reductions of ammonia-nitrogen, phosphorus, COD and the biochemical oxygen demand (BOD) of 61%, 84%, 50.5% and 61.6%, respectively (Kamaruddin et al., 2013). Bich et al. (1999) reported that *Chlorella vulgaris* was used in the treatment of rubber latex concentrate processing wastewater and that this microalga reduced the COD and total Kjeldahl nitrogen (TKN) by 93.4% and 79.3%, respectively. Another study carried out by Nordin et al. (1989) used high rate algal ponds (HRAP) to treat rubber effluent from an anaerobic digester, and the reductions in COD, BOD, NH_3-N and phosphorous reached 69.1%, 87.4%, 62.2% and 21.3%, respectively. In the HRAP, *Chlorella* was the predominant genus (Nordin et al., 1989).

Moderately polluted textile wastewater was previously reported to be treated using the microalga *Chlorella vulgaris*, with color and COD reductions of up to 69.9% and 75.7%, respectively (El-Kassas

and Mohamed, 2014). Another study found that this species could degrade 63-69% mono-azo dyes into simple aromatic compounds (Acuner and Dilek, 2004). Lim et al. (2010) investigated the treatment of textile wastewater using ten different strains of microalgae and found that *Chlorella vulgaris* was able to remove color from the wastewater. When cultured in a HRAP, color removal reached 50% along with high reductions in COD, PO_4^{3-}-P and NH_4^+-N (Lim et al., 2010).

Two wild type green algae such as *Micractinium* sp. and *Chlorella* sp. can also be grown in high nitrogen wastewater (mixture of sludge centrate and primary effluent wastewater). The extraction and analysis of extracellular polymeric substances (EPS) in both algal species during cultivation showed that *Micractinium* generated a higher amount of EPS-proteins than *Chlorella* (Wang and Park, 2015). This fact affects the anaerobic biodegradability and methane yield when these algae are anaerobically co-digested with waste activated sludge (WAS).

2.1.2. *Scenedesmus* genus

Food wastewater (FW), rich in nutrients including N, P, Ca, Fe, Al and total organic carbon (TOC) were also effectively used for microalgal cultivation (Ji et al., 2015a). The effect of FW supplementation on the biomass and lipid productivity of *Scenedesmus obliquus* cultivated in Bold's Basal Medium (BBM) was recently investigated by Ji et al. (2015a). They reported a

substantial increase in growth and lipid productivity with supplementation of 1% FW to BBM. Furthermore, the fatty acid methyl ester (FAME) analysis revealed that the palmitic and oleic acid contents increased by up to 8% with the addition of FW. They also noted that FW promoted algal autoflocculation due to the formation of inorganic precipitates at an alkaline pH (Ji et al., 2015a). Similarly, the biomass, lipid productivity and nutrient removal efficiency of *Scenedesmus obliquus* cultivated under mixotrophic conditions in municipal wastewater was reportedly enhanced when supplemented with FW and flue gas CO_2 (Ji et al., 2015b).

Shanab et al. (2012) demonstrated that out of three fresh water microalgal isolates selected for heavy metal tolerance studies, *Scenedesmus quadricauda* showed tolerance to heavy metals such as Hg^{2+}, Pb^{2+} and Cd^{2+} in up to 100 mg/L concentrations. Research on the applications of immobilized microalgal cells indicated that immobilized algal cells are more tolerant to heavy metal stress compared to free living cells (Shanab et al., 2012).

Scenedesmus sp. has also been widely reported with good carbon sequestration potential (Toledo-Cervantes et al., 2013). These studies obtained hydrocarbons from microalgal lipids for their conversion into sustainable biofuels as a substitute for fossil hydrocarbons. Furthermore, microalgae have significant applications in the production of valuable materials in the food and

pharmaceutical industries, producing a high value-added process in the bio-sequestration of CO_2 (Toledo-Cervantes et al., 2013).

Similar to bioconversion, some microalgae can also carry out the biosorption of textile wastewater. For instance, *Scenedesmus quadricauda* has been successfully employed as biosorbent to remove remazol brilliant blue R (RBBR) (Ergene et al., 2009; Fazal et al., 2018).

In a very recent study, microalgae digestate and secondary effluent were used to grow *Scenedesmus* sp. in a tertiary treatment using a 30 L closed photobioreactor for cultivation. The microalgae biomass, composed of *Scenedesmus* sp., reached and maintained a concentration of 1.1 g TSS/L during 30 days (Arias et al., 2018). A complete removal of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ and high nitrate and organic matter removals were achieved (58% $N\text{-}NO_3^-$ and 70% COD) with 8 days of HRT (Arias et al., 2018).

2.1.3. *Dunaliella salina*

A very recent study assessed the feasibility of the cultivation of *Dunaliella salina* in controlled-environment tertiary-treated municipal wastewater (Liu and Yildiz, 2018). *D. salina* was selected for its high beta carotene generation capacity and for being a halophilic species to protect our fresh water resources further through wastewater remediation. Nutrient analyses indicated that *D. salina* can significantly remove nitrate, ammonia, and phosphorus from municipal wastewater in the range of 45% to

88%. Among all combinations studied, optimal algal growth was observed at 30 ppt salinity level, with a 75% wastewater concentration (3:1 ratio of wastewater and saline water mixture, which is the growth medium). These findings concluded that *D. salina* has great capacity for nutrient uptake while providing high-value bioproducts (Liu and Yildiz, 2018).

Another study assessed the production rates of some native microalgae growing in media supplemented with algal digestate, urban wastewater or digested sludge. Very low production rates, or no growth, were measured when microalgae isolated from high salinity waters (*Dunaliella salina*) were used, suggesting that populations well adapted to extreme environmental conditions are not suitable candidates for growing in wastewater or anaerobic digestate (Fouilland et al., 2014).

2.1.4. *Nannochloropsis salina*

The potential for *Nannochloropsis salina* to be integrated with contaminated water sources was assessed for the concurrent production of a biofuel feedstock while providing an environmental service through bioremediation (Torres et al., 2017). Individual contaminants (As, Cd, Cr, Co, Cu, Pb, Ni, Hg, Se, and Zn) at various concentrations ranging from a low concentration (1X) to higher concentrations (10X, and 40X) found in contaminated systems (mine tailings, wastewater treatment plants, produced water) were introduced into growth media. Biological growth

experimentation was performed in triplicate at the various contaminant concentrations and at 3 different light intensities. Results showed that baseline concentrations of each contaminant slightly decreased biomass growth to between 89% and 99% of the control with the exception of Ni which dramatically reduced growth. Increased contaminant concentrations resulted in progressively lower growth rates for all the contaminants tested. Lipid analysis showed most baseline contaminant concentrations slightly decreased or had minimal effects on lipid content at all light levels. Trace contaminant analysis on the biomass showed that Cd, Co, Cu, Pb, and Zn were sorbed by the microalgae with minimal contaminants remaining in the growth media, which illustrated the effectiveness of microalgae to bioremediate these contaminants when levels are sufficiently low and to not detrimentally impact productivity. The microalgae biomass was less efficient in the sorption of As, Cr, Ni, and Se (Torres et al., 2017).

Another study revealed that metal levels in municipal wastewaters were unlikely to inhibit algal growth and lipid production at least by metals which are tolerant to microalgae like *Nannochloropsis salina*. Cells grew without inhibition in treated municipal wastewater or centrate derived from wastewater treatment at additions of up to 75% v/v in their normal growth medium minus nitrogen and phosphorus (Dong et al., 2014).

2.1.5. *Botryococcus braunii*

Botryococcus braunii is a microalga which is regarded as a potential source of renewable fuel because of its ability to produce large amounts of lipids that can be converted into biodiesel. Agro-industrial by-products and wastes are of great interest as cultivation medium for microorganisms because of their low cost, renewable nature, and abundance. Two strategies for the low-cost production of *B. braunii* biomass with high lipid content were performed: (i) mixotrophic cultivation using molasses, a cheap by-product from the sugar cane plant as a carbon source, and (ii) photoautotrophic cultivation using nitrate-rich wastewater supplemented with CO_2 as a carbon source. Mixotrophic cultivation added with 15 g/L molasses produced a high amount of biomass at 3.05 g/L with a high lipid content of 36.9%. The photoautotrophic cultivation in nitrate-rich wastewater supplemented with 2.0% CO_2 produced a biomass of 2.26 g/L and a lipid content of 30.3%. The benefits of this photoautotrophic cultivation are that this cultivation would help to reduce the accumulation of atmospheric carbon dioxide and more than 90% of the nitrate could be removed from the wastewater. When this cultivation was scaled up in a stirred tank photo bioreactor and run with the semi-continuous cultivation regime, the highest microalgal biomass of 5.16 g/L with a comparable lipid content of 32.2% was achieved (Yeesang and Cheirslip, 2014).

To understand the potential of using swine lagoon wastewater to cultivate *Botryococcus braunii* for biofuel production, the growth characteristics of *B. braunii* 765 cultivated in aerated swine lagoon wastewater (ASLW) without sterilization and pH adjustment were investigated. The results showed that the alga strain could maintain a competitive advantage over the 26-day cultivation. The highest dry biomass of alga grown in ASLW was 0.94mg/L at day 24, which was 1.73 times that grown in a BG 11 medium, an artificial medium normally used for *B. braunii* cultivation. And the algal hydrocarbon content was 23.8%, which was more than twice that in the BG 11 medium. Additionally, after the 26-day cultivation period, about 40.8% of TN and 93.3% of TP in ASLW were removed, also indicating good environmental benefits of algal bioremediation (Liu et al., 2013).

A study was conducted to evaluate the possibility of using wastewater from a soybean curd manufacturing plant as a growth promoter of *Botryococcus braunii* strain BOT-22. Soybean curd wastewater (SCW) was added to a AF-6 medium to set final concentrations at 0% (control), 1%, 2%, 5%, and 10% (v/v). The growth and hydrocarbon production observed in the cultures with 1% and 2% SCW were significantly higher than that observed in the control. It was postulated that proteins and/or reducing sugars in SCW could enhance the growth (Yonezawa et al., 2012).

2.1.6. *Micractinium* genus

The strain *Micractinium* sp. IC-76 was grown in municipal wastewater and showed a biomass productivity of 37.1 ± 4.1 mg/(L d) and a lipid content of 36.2 ± 0.1%, with a total content of saturated and monounsaturated fatty acids of 71.9%. The efficiency of nitrogen ($N\text{-}NH_4^+$) and phosphorus ($P\text{-}PO_4^{3-}$) removal was 96.4 ± 0.7 and 77.8 ± 5.6%, respectively. The strain *Micractinium* sp. IC-76 in the stationary phase of growth showed a significant difference in carbohydrate metabolism, especially sucrose concentration. High lipid induction during cultivation in wastewater was also driven by changes in the biosynthesis of amino acids, fatty acids and the tricarboxylic acid cycle (Piligaev et al., 2018).

Micractinium sp. Embrapa|LBA32 presented vigorous growth in a light-dependent manner in undiluted vinasse under non-axenic conditions. Microalgae strains presented higher biomass productivity in vinasse-based media compared to standard BBM in cultures performed using 15 L airlift flat plate photobioreactors. Chemical composition analyses showed that proteins and carbohydrates comprise the major fractions of algal biomass. Glucose was the main monosaccharide detected, ranging from 46% to 76% of the total carbohydrate contents according to the culture media used (Santana et al., 2017).

2.2. Haptophytes: *Isochrysis galbana*.

A recent study investigates the capacity of *Isochrysis galbana* in the bioremediation of aquaculture wastewater from a grey mullet Mugil cephaluser. The experiment was conducted in batch conditions for 7 days using completely mixed bubble column photobioreactors. After two days, *I. galbana* removed 32% and 79% of dissolved inorganic nitrogen and dissolved inorganic phosphorus, respectively (Andreotti et al., 2017).

It has been also reported that *Isochrysis galbana* cultured in open ponds has fatty acids and a high protein content which are suitable for animal nutrition (Udaiyappan et al., 2017).

2.3. Cyanobacteria

2.3.1. *Arthrospira platensis*

Phosphorus can be recycled from wastewater via microalgal cultivation and provided to crop plants in the form of microalgal biofertilizers. Guldhe et al. (2017) reported filamentous cyanobacteria *Arthrospira platensis* cultivated in aquaculture wastewater as algal biofertilizer for the leafy vegetables Arugula (*Eruca sativa*), Bayam Red (*Amenranthus gangeticus*) and Pak Choy (*Brassica rapa ssp. Chinensis*). In their study, *Arthrospira platensis* biomass showed lower amounts of NPK, while amounts of iron, magnesium, calcium and zinc were found to be higher in

algal biomass when compared with chemical fertilizer (Triple Pro 15-15-15).

Microalgae are a rich source of proteins, pigments and omega fatty acids and thus find application in human as well as animal feed production. *Arthrospira platensis* is one of the dominant species of microalgae used in the health food industry (Suganya et al., 2016).The omega fatty acids from this microalga are used as human food and animal feed supplements. Phang et al. (2000) found that the biomass composition of *Arthrospira* cultured in a high-rate algal pond for the treatment of sago starch processing wastewater can be used as high-quality animal feed, especially in the aquaculture industry. During the mentioned treatment of sago processing wastewater using *Spirulina*, COD, PO_4^{3-}-P and NH_4^{+}-N reductions of 94%, 93% and 99% respectively were achieved (Phang et al., 2000).

Zainal et al. (2012) reported that *Arthrospira platensis* was able to treat wastewater containing heavy metals and removed manganese by 84.9%; chromium by 83.8%; arsenic by 71.4%; nickel by 61.9%; zinc by 55%; copper by 52.8% and iron by 45.1%.

Similar to bioconversion, microalgae could also carry out the biosorption of textile wastewater. For instance, *Arthrospira platensis* was used as a biosorbent to remove reactive red 120 (RR-120) from its aqueous solution. It achieved the maximum

biosorption capacity of 482.2 mg/g removing 97% RR-120 from the solution (Cardoso et al., 2012).

2.3.2. *Oscillatoria tenuis*

The performance of *Oscillatoria tenuis* to remove nitrogen, phosphorus and COD from secondary effluents of municipal domestic wastewater was investigated in batch experiments. *Oscillatoria tenuis* had a biomass productivity of 150 mL/(L·d), a removal rate of NH_4^+-N of 96.1%, and total phosphorus and COD removal efficiencies of 82.9% and 92.6%, respectively, within 7 days at an aeration rate of 1.0 L/min (Cheng et al., 2018).

At the same time, *Oscillatoria tenuis* showed its capacity to remove reactive dyes from textile wastewater. This species degraded azo dyes into simple aromatic amines and decolorized dye wastewater (Fazal et al., 2018).

2.4. Binary and mixed culture systems

Maintaining the uni-algal system requires a super clean environment, which can be attained under laboratory conditions only. In the outdoor cultivation of microalgae, it is almost impossible to maintain a uni-algal system. If so, it requires a lot of expertise and skills. Moreover, the biomass productivity of the uni-algal system is limited because of suppressed metabolic activity during night time or dark periods. Alternatively, heterotrophic microalgae are used, which are less sensitive to photoperiods, grow

fast, and return high biomass yields. However, a significant amount of CO_2 is produced during oxidative metabolism, which remains un-used and is released into the environment. This CO_2 can be further utilized by employing autotrophic microalgae in the cultivation matrix. Therefore, the concept of a binary culture system arises (Rashid et al., 2018). Binary culture is considered superior to the uni-algal system in several different ways: binary culture can use wastewater as a nutrients source without sterilization unlike in single systems; microalgae observe a low level of contamination in binary culture since bacteria protect those invading pathogens; microalgae with increased growth rate would decrease the cultivation time and reduce overall cost; binary culture also aids in bioflocculation and lipid induction, etc. (Rashid et al., 2018).

Specie selection is crucial for the success of microalgae cultivation in wastewater. Combining different species with varying metabolic potential would provide robustness to fluctuations in environmental factors and wastewater compositions, thereby giving more stability to the system. For instance, the potential application of microalgae consortia (*Chlorella* sp., *Scenedesmus* sp., *and C. zofigiensi*) compared to monoculture (*Chlorella* sp.) for the treatment of dairy wastewater was evaluated by Qin et al. (2016). They reported a significantly higher COD removal (57-62%) and phosphorous removal (91-96%) by microalgae consortia compared to the monoculture of *Chlorella* sp.

Furthermore, FAME profiles indicated that lipids produced from the microalgae consortia cultivation system were more suitable for biodiesel production (Qin et al., 2016).

In a very recent study (Huy et al., 2018), a mixed microalgae consortium (highly dominated by *Chlorella* species and small portions of *Scedesmus* sp.) was cultivated using digestate (D), animal manure (AM) and textile wastewater (TW) as growth medium providing mainly N (nitrogen) and P (phosphorous) sources without any extra nutrient addition. After a cultivation period of 13 days, P was completely removed (100%), however, N was still remaining and the removal rates of 70.1, 72.3 and 16.7% for TW, AM and D, respectively, were achieved. The peak growth rate and biomass production of 0.419 d^{-1} and 0.4 g/L (in terms of volatile solids, VS) were achieved using TW as growth medium (Huy et al., 2018).

3. Use of microalgae for biogas production through anaerobic digestion

Anaerobic digestion is a series of biological processes in which microorganisms break down biodegradable material in the absence of oxygen. The end-products of anaerobic digestion are biogas and a digestate. Recently, algal biomass has been identified and developed as a renewable fuel source, and the growth of algal biomass for methane production has been increased.

The first study concerning the anaerobic digestion of microalga was carried out by Goluke et al. (1957). *Secenedesmus* sp. and *Chlorella* sp. were used as substrates for anaerobic digestion under different conditions. The authors finally concluded that microalgae have a relatively low digestibility due to the slowly biodegradable cell wall. Recently, one of the first studies about using algal biomass in anaerobic digestion was carried out by De Schamphelaire and Verstraete (2009). This work consisted of designing a closed loop where algal biomass was used to obtain biogas. The maximum methane yield reached was 65 mL/day. More recently, in 2013 Torres et al. (2013) defined the ideal microalgae for anaerobic digestion as a large cell microalga with a very thin cell wall or lacking it, with a high growth rate in non-sterile medium and great resistance against natural pollutants. In one of the latest studies on the anaerobic digestion of microalgae, the authors pointed out the main limitations during the anaerobic digestion of microalgae, noting the low degradability of the cell wall, ammonium toxicity and salinity as the main inhibitors of anaerobic digestion (González-González et al., 2018).

However, the use of microalgae as co-substrate is an approach to dilute complex compounds and balance the C/N ratio. Co-digestion has several advantages such as adjusting the C/N ratio, nutrients and inhibitor compounds (Hartmann and Ahring, 2005). Ajeej et al. (2015) also reported the increased activity of methanogenic microorganisms, a decreased anaerobic digestion inhibition by

ammonium and even an increase in cellulose activity when carbon-rich materials were added. Taking into account that the C/N ratio of the microalgal biomass is around 10/1 (Geider and La Roche, 2002), the microalgae biomass can be considered as a suitable feedstock for carbon-rich substrates (Heerenklage et al., 2010).

The main microalgae used for co-digestion have been:

3.1 Chlorophytes

3.1.1. *Chlorella* genus

Ehimen et al. (2009) added lipid-extracted *Chlorella* biomass resulting from microalga diesel production to glycerol (main by-product formed during the transesterification process) and observed an increase in the methane yield of 50% when compared with the digestion of residual biomass alone.

Wang et al. (2013) used the biomass of microalga *Chlorella* sp. Grown in lab culture for co-digestion with WAS. The batch experiments were carried out under mesophilic conditions with a working volume of 100 mL. Different volumes of algae and WAS were added to the digester. They experimentally proved that the addition of WAS improved the anaerobic digestion of the microalga *Chlorella*, producing 73-79% more methane than single microalga digestion. Similar results were obtained by Li et al. (2017a), who co-digested *Chorella* sp. with chicken manure in

batch experiments. The co-digestion enhanced the methane production obtained during the single digestion of chicken manure and *Chlorella* sp. by 14.20 and 76.86%, respectively. By contrast, Retfalvi et al. (2016), using the same C/N ratio, but pre-treating the microalga, did not observe any positive effects on methane production.

Beltran et al. (2016) assessed the co-digestion of *Chlorella sorokiniana* with WAS. Different co-digestion mixtures were tested in biochemical methane potential (BMP) tests under mesophilic conditions. The highest methane yield obtained was 442 mL CH_4/g VS for the mixture 75% WAS and 25% microalga. This value was 22% and 39% higher than that obtained in the anaerobic digestion of the sole substrates, WAS and microalga, respectively. This mixture clearly improved anaerobic digestion by ensuring its viability, suitability and efficiency.

Rusten and Sahu (2011) co-digested *Chlorella* sp. biomass and wastewater sludge (pre-treated sludge liquor).The specific methane gas production (mL CH_4/g VS $_{fed}$) was not increased when compared to the anaerobic digestion of wastewater sludge alone. The co-digestion process achieved between 65 and 90% of specific methane gas production for sludge liquor depending on the HRT, temperature of incubation and pre-treatment of algae biomass. However, this study indicated that tested microalga could be cultivated in reject water to remove nitrogen and phosphorus from the sludge liquor.

In a recent study, Mahdy et al. (2017) investigated the anaerobic co-digestion of *Chlorella vulgaris* and manure. They used five different mixtures in a batch mesophilic experiment. The percentage 80:20 microalga:manure produced 431 mL CH_4/g VS, while the methane yield of the single microalga produced 415 mL CH_4/g VS. Despite the high ammonium levels (3.7-4.2g NH_4^+-N /L), using ammonia tolerant inoculums resulted in a relatively high methane yield.

According to Li et al. (2017b), *Chlorella* 1067 was cultivated in a chicken manure-based digestate and the resulting algae biomass was used as co-substrate with chicken manure in anaerobic co-digestion. The growth of microalga in manure-based digestate recycled about 91% of the total nitrogen and 86% of the soluble organic phosphorous. During co-digestion, the highest methane production was 238.71 mL CH_4/g VS, obtained at the mixing ratio of 8:2 (chicken manure to *Chlorella* 1067 according to the VS).

3.1.2. *Scenedesmus* genus

Ramos-Suarez and Carreras, 2014) described *Scenedesmus* sp. biomass as a non-suitable substrate for anaerobic digestion due to its low degradability and low methane production. In contrast, during their investigations, they used the biomass of microalga as co-substrate with *Opuntia maxima* cladodes. Bioreactors were used to grow *Scenedesmus* sp. and the biomass was co-digested with different percentages of cladodes of one or two years of age in

order to avoid an increase in lignocelluloses. C/N ratios from 6.0 to 51.3 were used, proving that co-digestion improved methane yield and kinetics compared to the mono-digestion of both substrates. The best mixture turned out to be the C/N ratio of 15.6. The methane yield for this mixture was 233.6±16.4 mL CH_4/g VS and was increased by 66.4% and 63.9% when compared to *Scenedesmus* sp. biomass and *Opuntia maxima* when digested alone.

Astals et al. (2015) assessed the co-digestion of pig manure and *Scenedesmus* sp. with and without the extraction of intracellular algal co-products. Proteins and/or lipids were extracted from *Scenedesmus* sp. This process increased methane yield by 29-37% compared to raw microalga biomass. Co-digestion experiments showed a synergy effect between pig manure and raw microalga that increased raw algae methane yield from 163 to 245 mL CH_4/g VS. A similar synergy effect was not observed when algal residues were co-digested with pig manure.

Arias et al. (2018) used microalga digestate and secondary effluent to grow microalga in a tertiary wastewater treatment, and then the microalga biomass was co-digested for biogas generation. The algal biomass was mainly composed of *Scenedesmus* sp. The algae biomass and the WAS were pre-treated by autohydrolysis reaching 11.4% and 25.7% of solubilization, respectively. The solubilization of *Scenedesmus* biomass was lower than the solubilization of WAS after pre-treatment and S*cenedesmus* has

been reported to have a complex multilayer cell wall (Tukaj and Bohdanowicz, 1995). After pre-treatment both substrates were co-digested in different proportions. The maximum methane yield obtained was 204 mL CH_4/g VS for the anaerobic digestion of 100% WAS. On the other hand, the methane yield of the anaerobic digestion of 100% microalga exhibited a 64% lower methane production and reached 134 mL CH_4/g VS. The mixture 20% microalga-80% WAS produced 187 mL CH_4/g VS while the mixture 50% microalgae-50% WAS produced 162 mL CH_4/g VS and the mixture 80% microalga-20% WAS produced 132 mL CH_4/g VS. The results showed neither positive nor negative synergies between substrates, meaning that co-digestion did not improve microalga anaerobic biodegradability (Arias et al., 2018).

3.1.3. *Dunaliella salina*

According to Fernández-Rodríguez et al. (2014), the addition of olive mill solid waste (OMSW) to *Dunaliella salina* biomass resulted in the improvement in methane yield and biodegradability of OMSW compared to the anaerobic digestion of the sole substrates. The experiment was carried out in batch and different percentages of OMSW and *Dunaliella salina* biomass were tested. The highest biodegradability was found for the co-digestion mixture 50% OMSW-50% *Dunaliella salina*. Nevertheless, the maximum methane production, 330 mL CH_4/g VS, and the highest methane production rate were obtained for the co-digestion mixture

75% OMSW-25% *Dunaliella salina*, keeping a C/N ratio close to 26.7.

3.1.4. *Nannochloropsis salina*

Another approach to enhancing biogas production from microalga through co-digestion was assessed by Schwede et al. (2013). Corn silage is one is the most common waste products produced around all over the world. Corn silage is characterized as being a lignocellulosic residue and very difficult to digest by anaerobic digestion (Oleskowicz-Popiel et al., 2008). The experiment carried out by Schwede et al. (2013) reached a high methane yield using *Nannochloropsis salina* as a co-substrate of corn silage. The mixture balanced the nutrient composition due to the corn silage providing mainly carbon and the microalga providing nitrogen, which helped to balance the C/N ratio from 65 (*Nannochloropsis salina*) or 32.6 (corn silage) to 21.2. This mixture reached 9% more methane than that obtained in the anaerobic digestion of the corn silage alone.

3.1.5. *Botryococcus braunii*

Neumann et al. (2015) reported that the anaerobic co-digestion of lipid-spent *Botryococcus braunii* (LSBB) with WAS and glycerol showed no significant increase in BMP when mixing these substrates. However, the kinetic constant of the mixture 25% WAS-75% LSBB was much higher than those obtained for WAS

and LSBB alone. The mixture 10% glycerol- 90% LSBB did not show a higher kinetic constant or methane production. The authors concluded that the application of different cultivation procedures, lipid extraction methods and anaerobic conditions will result in different microalga biomass compositions and characteristics, which affect the productivity of microalgal methane.

3.1.6. *Micractinium* genus

Wang et al. (2015) applied WAS to the digestion of microalga biomass consisting of *Micractinium* sp. The algae biomass was grown in high-nitrogen wastewater (mixture of sludge centrate and primary effluent wastewater). The microalga showed a good ability for nutrient removal throughout the growth. The co-digestion of microalga biomass and WAS improved the solubilization efficiency as well as the biodegradability of the microalgae. The methane yield obtained for the microalga was 209 mL/g VS. The co-digestion of algae with WAS improved the volatile solid reduction, the solubilization efficiency of the algae, and their biogas yield. However, the methane production of the WAS alone showed no improvement.

3.1.7. *Selenastrum capricornotum* (Chlorophyta) and *Isochrysis galbana* (Haptophyta)

Isochrysis galbana and *Selenastrum capricornutum* were co-digested with sewage sludge under mesophilic (33 ºC) and

thermophilic (55 ºC) conditions (Caporgno et al., 2015). Under mesophilic conditions the anaerobic digestion of sewage sludge produced 451±12 mL biogas/g VS. The microalga *Isochrysis galbana* produced 439 mL biogas/g VS and *Selenastrum capricornutum* produced 271 mL biogas/g VS. When a substrate mixture was fed, biogas production showed quite similar values for all experiments, regardless of the sludge to microalga ratio in the mixture. The average biogas production was 440±25 mL biogas/g VS. So, microalga and sewage sludge co-digestion did not improve biogas yield in comparison with individual digestions of both substrates under mesophilic conditions. Under thermophilic conditions, the biogas production of *Isochrysis galbana* was 261±11 mL biogas/g VS and the production of *Selenastrum capricornutum* was 185±7 mL biogas/g VS. The amount of methane decreased by 40.5% and 31.7% for *Isochrysis galbana* and *Selenastrum capricornutum*, respectively, compared to their biogas production at 33 ºC. The increase in temperature had a negative influence on microalga digestion. However, temperature had a huge beneficial effect on sewage sludge. The production of biogas reached 566±5 mL biogas/g VS, indicating that 25.5% more biogas was produced by increasing temperature. The experiment presented similar tendencies, the higher the volatile solid, the lower the biogas production.

3.2 Cyanobacteria

3.2.1. *Arthrospira platensis*

Arthrospira platensis was characterized as having a high level of protein and, therefore, a high nitrogen content (Tokuşoglu and üUnal, 2003). Biomass with a high nitrogen content could be used as co-substrate with high-carbon content substrates (Herrmann et al., 2016). This study investigated the co-digestion of *Arthrospira platensis* with barley straw, beet silage and brown seaweed at a C/N ratio of 25, the optimal ratio for anaerobic digestion (Li et al., 2011). The experiments were carried out in batch and semi-continuous systems. The C/N ratio of the substrates were 4.3, 145.5, 41.7 and 28.7 for *Arthrospira platensis*, barley straw, beet silage and seaweed *Laminaria digitate*, respectively. The methane productions during the batch experiments were 357.1, 196.8, 393.5 and 306.5 mL_N/gVS for *Arthrospira platensis*, barley straw, beet silage and seaweed *Laminaria digitate*, respectively. The co-digestion 45% *Arthrospira platensis*- 55% beet silage produced 360.9 mL_N/gVS. The co-digestion 85% *Arthrospira platensis*- 15% barley straw produced 347.8mL_N/gVS and the best co-digestion mixture of *Arthrospira platensis*-*Laminaria digitate* (15%-85%) produced 311.5 mL_N/gVS. Mono-digestion of *Arthrospira platensis* led to high methane yields in the semi-continuous mode, but only at low organic rates of 1.0 g VS/L·d. Co-digestion with carbon rich substrates had a positive effect on process stability. The highest

biogas production occurred during co-digestion of microalga with beet silage. The best process stability was found at an organic loading of 4.0 g VS/L·d during co-digestion with the seaweed *Laminaria digitate* (Herrmann et al., 2016).

Arthrospira platensis was co-digested with WAS in batch and in semi-continuous systems (Varol and Ugurlu, 2016). During the batch tests the system reached 89-93% volatile solid reduction. The biogas production was between 210 and 260 mL CH_4/g VS. In the continuous studies a two-phase anaerobic digestion system was investigated. The system achieved 60% of volatile solid reduction with 525 mL biogas/gVS·d. The co-digestion of *Arthrospira platensis* and sewage sludge improved biogas production and volatile solid reduction. The best mixture was 66.6% WAS and 33.3% *Arthrospira platensis* based on volatile solids. The maximum methane production was 640 mL biogas/g VS·d with a 62.5% reduction in volatile solids. The methane content in the biogas was 77%.

3.2.2. *Oscillatoria tenuis*

Cheng et al. (2018) carried out batch experiments to investigate the performance of *Oscillatoria tenuis* to remove nitrogen, phosphorus and COD and from the secondary effluents of municipal domestic wastewater. The potential of biogas production was also investigated by applying the co-digestion of *Oscillatoria tenuis* with pig manure. *Oscillatoria tenuis* had a good biomass

productivity which ranged from 104 to 150 mg/L·d, and was beneficial for the subsequent anaerobic digestion. A maximum methane yield of 191 mL CH_4/g VS was achieved through co-digestion of this microalga with pig manure at a mixing ratio of 2.0.

3.3 Binary culture system

3.3.1. *Scenedesmus* genus +*Chlorella* genus

Zhen et al. (2016) used a mixed microalgae culture of *Scenedesmus* sp. and *Chlorella* sp., which were co-digested with food waste in a batch system under mesophilic conditions. The results showed that supplementing food waste with microalga significantly improved the performance of microalga digestion. The highest methane yield achieved was 639.8±1.3 mL/g VS, which was reached at a microalga:food waste ratio of 0.2:0.8,obtaining a 4.99 fold increase with respect to microalgae alone (106.9±3.2 mL/g VS).

3.3.2. Microalgae + bacteria

Solé-Bundó et al. (2017a) grew microalgae biomass in wastewater and subsequently the algae-bacteria biomass was co-digested with wheat straw. Batch systems were used for testing different substrate percentages (20-80%, 50-50% and 80-20%, microalgae-wheat straw, respectively, on a volatile solid basis). The highest synergies in degradation rates were observed by adding

at least 50% wheat straw. Therefore, the co-digestion of 50% microalgae biomass-50% wheat straw was further investigated in mesophilic semi-continuous lab-scale reactors. The results showed that the methane yield was increased by 77% in the co-digestion compared to microalgae biomass mono-digestion.

Table 1. summarizes the different microalgae and co-substrates tested in anaerobic co-digestion processes including the improvement in the methane yields observed. (C: carbon, N: nitrogen, WAS: waste activated sludge, OMSW: olive mill solid waste, *: not available)

Microalga	Co-substrate	Conditions	Improvement in methane yield (%)	Reference
Lipid-extracted *Chlorella* biomass	Glycerol C/N =12.44	Laboratory scale, continuously stirred tank reactor, at mesophilic temperature	>50 (compared to microalga)	Ehimen et al., 2009
***Chlorella* sp. (4%)**	WAS (96%)	Batch at mesophilic temperature	73-79 (compared to microalga)	Wang et al., 2013
***Chlorella* 1067 (20%)**	Chicken manure (80%)	Batch experiments	77 (compared to microalga)	Li et al., 2017a
Pretreated *Chlorella* sp.	Chicken manure	Batch experiments	no positive effect	Rétfalvi et al., 2016

(80%)	(20%)			
***Ch. sorokiniana* (25%)**	WAS (75%)	Batch at mesophilic temperature	39 (compared to microalga)	Beltrán et al., 2016
***Chlorella* sp. (12%)**	Wastewater sludge (88%)	Batch at mesophilic temperature	12 (compared to single substrate)	Rusten et al., 2011
***Chl. vulgaris* (80%)**	Manure (20%)	Batch at mesophilic temperature	3.8 (compared to microalga)	Mahdy et al., 2017
***Scenedesmus* sp. (25%)**	*Opuntia maxima* cladodes (75%)	Batch at mesophilic temperature	66.4 (compared to microalga)	Ramos-Suárez and Carreras, 2014
***Scenedesmus* sp. (15%)**	Pig manure (85%)	Batch at mesophilic temperature	50.3 (compared to microalga)	Astals et al., 2015
***Scenedesmus* sp. (20%)**	WAS (80%)	Batch at mesophilic temperature	39.5 (compared to microalga)	Arias et al., 2018

Dunaliella salina (25%)	OMSW (75%)	Batch at mesophilic temperature	3 (compared to single substrate)	Fernández-Rodríguez et al., 2014
Nannochloropsis salina (16.6%)	Corn silage (83.4%)	Batch at mesophilic temperature	6 (compared to microalga)	Schwede et al., 2013
Lipid-spent *Botryococcus braunii*	WAS and glycerol	Batch at mesophilic temperature	No positive effect	Neumann et al., 2015
***Micractinium* sp. (79%)**	WAS (21%)	Batch at mesophilic temperature	10 (compared to microalga)	Wang and Park, 2015
Isochrysis galbana* and *Selenastrum capricornutum	Sewage sludge	Batch at mesophilic and thermophilic temperature	no positive effect	Caporgno et al., 2015
***Arthrospira platensis* (85%)**	Barley Straw (15%)	Batch at mesophilic temperature	76.7 (compared to single substrate)	Herrmann et al., 2016
***Arthrospira platensis* (45%)**	Beet Silage (55%)	Batch at mesophilic temperature	1.1 (compared to microalga)	Herrmann et al., 2016

***Arthrospira platensis* (15%)**	*Laminaria digitate* (85%)	Batch at mesophilic temperature	1.6 (compared to single substrate)	Herrmann et al., 2016
***Arthrospira platensis* (33.3%)**	WAS (66.6%)	Two Stages Semi-continuous	32.5 (compared to microalga)	Varol and Ugurlu, 2016
***Oscillatoria tenuis* (66.6%)**	Pig Manure (33.3%)	Batch at mesophilic temperature	*	Cheng et al., 2018
***Scenedesmus* genus + *Chlorella* genus (20%)**	Food Waste (80%)	Batch at mesophilic temperature	498.5 (compared to microalga)	Zhen et al., 2016
***Chlorella* sp. + some *Monoraphidium* sp. (50%)**	Wheat Straw (50%)	Batch at mesophilic temperature	77 (compared to microalga)	Solé-Bundó et al., 2017a

4. Microalgae growth in anaerobic digestates

4.1. Physico-chemical characterisation of digestates

The anaerobic digestate studied by Solé-bundó et al. (2017b) presented low dry matter content (3%); and these digestates can therefore be treated as liquids that could be directly spread onto soil as fertilizer. A problem arises when transportation is required and moisture reduction could be necessary. Anaerobic digestate from microalgae co-digestion was observed to present better water release than the digestate from single microalga digestion.

Other parameters that could have a negative impact on soil (pH, electrical conductivity and volatile fatty acids) were lower in the co-digestion digestates, indicating that microalgae co-digestion resulted in a more stable digestate.

In general, among the bibliography, anaerobic digestates from agro-food industries presented higher organic contents than those from microalgae digestion (Teglia et al., 2011), which could be explained due to organic matter mineralization during anaerobic digestion processes. The use of microalgae as co-substrate in the digester reduce the VS/TS ratio when compared with microalga alone (from 53-54% to 47%) due to the better biodegradability of the organic compounds of the co-substrate.

In order to evaluate the feasibility of these anaerobic digestates as fertilizers some elemental nutrients were evaluated. The total nitrogen content was higher in the non-co-digested microalgae (80

g/kg TS and 56g/kg TS); although the $N\text{-}NH_4^+$/TKN ratio, which represents the soluble mineral nitrogen fraction, only varied from 30.9 to 33.8% among all digestates. Moreover, the C/N ratio was low across the board, which means that in each case the nitrogen content is too high for its use as fertilizers; although it could be used as soil amendment. This problem could be sorted out by using a high carbon content co-substrate like OMSW or corn silage. Phosphorous and potassium were found slightly higher in the digestates from non-co-digestion; although in each case the content was relatively low and similar to other anaerobic digestates reported in the literature. Calcium, magnesium and sodium were also analyzed and no difference was observed among the different digestates Solé-bundó et al. (2017b).

On the whole, the anaerobic digestate from microalgae co-digestion presented better suitability for nutrient supply in soil due to its low C/N ratio, which could be enhanced by using a co-substrate with a higher carbon content.

4.2. Microalgae growth in digestates

The anaerobic digestion of biomass produces a high nutrient digestate which is usually used as crop fertilizer, and also could be used as a nutrient supply for microalgae growth in order to reduce the use of external sources of nitrogen and phosphorous (Bjornsson et al., 2013). Moreover, wastewaters and other biomass present a reduction in suspended solids and color, better degradability, a

more stable pH and a reduction in pathogens after the anaerobic digestion process which could enhance microalgae growth when compared to the non-digested biomass.

The main factors that could affect the microalgae growth in anaerobic digestates are the nitrogen and phosphorous contents as well as the pH profile. pH could be increased due to active photosynthesis or insufficient CO_2 supply, which could provoke a $N\text{-}NH_4^+$ disappearance through gas stripping and a $P\text{-}PO_4^{3-}$ precipitation when the medium presents a high concentration of Ca^{2+} (De la Nüe and Basséres, 1989). Thus, when the pH of the medium is increased due to microalgae activity, nitrogen and phosphorous depletion do not necessarily mean an increase in biomass. Moreover, it has been reported that an ammonia concentration higher than 2 mM, when pH exceeds 8.1, presented a toxic effect on algae growth (Abeliovich, 1980). Regarding phosphorous content, it has been reported that 5 mg P/L was sufficient for adequate algae growth when the N/P ratio was around 15, although other studies suggested that N should be the limiting factor (De la Nüe and Basséres, 1989).

On the other hand, the organic load in these anaerobic digestates is reduced after microalgae cultivation. Nitrogen and phosphorous could be completely removed when the conditions are optimum and COD reduction could reach 44-85% depending on culture conditions and microalgae species (De la Nüe and Basséres, 1989).

4.2.1 Chlorophytes

4.2.1.1. Chlorella genus

An early study used different microalgae cultivated in swine manure anaerobic digestate diluted with tap water (0.6 – 3.0%) in order to evaluate its effect on microalgae growth. *Chlorella* sp. was the only species that presented pH stability (pH = 8.5 during 8 days) which indicated that the nitrogen removal was directly related to biomass production. Regarding temperature conditions, *Chlorella* sp. did not show any difference in biomass yield when the temperature was raised from 10 ºC to 20 ºC. COD reduction in the anaerobic digestate reached 60%. The best conditions for the highest concentration (41 mg dry wt/L·d) were 20 ºC and a manure concentration of 2% (De la Nüe and Basséres, 1989).

4.2.1.2. Parachlorella kessleri

Parachlorella kessleri was cultivated (12 days; 25 ºC; air flow: 0.5-1 L/min; illumination: 200 μmol/m^2·s) in the anaerobic digestate derived from the co-digestion of end-of-life dairy products with a given mixture of agro-industrial wastes (Koutra et al., 2017). Prior to the growth of algae, the anaerobic digestate was filtered, diluted (2%-10%) and then split into two different samples, one sterilized and the other not. Under the best conditions (2% dilution) *P. kessleri* presented a biomass yield of 270 mg/L, regardless of the use of sterilized or non-sterilized anaerobic

digestate. Moreover, according to the nutrient removal, the nitrogen depletion (up to 100%) and the phosphorous reduction (93.4%) were higher when the anaerobic digestate was sterilized and diluted by up to 2%. Nevertheless, the maximum COD removal (33.3%) was achieved with the non-sterilized anaerobic digestate and a higher dilution (10%). Regarding the fatty acid accumulation, after 25 days of growth, the concentration observed (31.1% of dry weight) was higher than in the control essay (19.6% dry weight).

4.2.1.3. Scenedesmus genus

De la Noüe et al. (1989) studied the growth of different microalgae in swine manure anaerobic digestate diluted with tap water (0.6 – 3.0%). The results showed that *Scenedesmus obliquus* presented a response to high temperature which could be a problem for outdoor work. This microalga was able to reduce the COD content of the anaerobic digestate by up to 85% with a microalga concentration of 57 mg dry wt/L·d at 20 ºC and with a manure concentration of 2% after 15 days.

In a different study *S. obliquus* was cultivated in the above mentioned conditions (Koutra et al., 2017). Under the best conditions (2% dilution), *S. obliquus* presented a biomass yield of 231 mg/L, regardless of the use of sterilized or non-sterilized anaerobic digestate. Moreover, according to the nutrient removal, the nitrogen depletion was higher (up to 100%) when the anaerobic digestate was sterilized and diluted by up to 2%. Nevertheless, the

phosphorous reduction was higher (92.5%) when the anaerobic digestate was not sterilized, and the maximum COD removal (53.7%) was achieved with the non-sterilized anaerobic digestate and a higher dilution (10%). The fatty acid accumulation, (26.6% dry weight), was higher after 25 days of growth than in the control essay (24.5% dry weight).

Different anaerobic digestates from microalgae biomass co-digestion with swine and cow manure and vegetable wastes were selected for the growth of *Scenedesmus* sp. AMDD at 22 ºC (Bjornsson et al., 2013). Nitrogen was adjusted to 1.5 mM (NH_3-N) with deionized water and different phosphorous concentrations were evaluated. Moreover, digestates were filtered to reduce the bacterial load. This study showed that the use of an anaerobic digestate from the co-digestion of microalgae biomass presented a good microalga growth rate. Animal manure digestate without co-digestion did not produce a complete nitrogen removal, which was improved when Mg^{+2} was added in the media growth. This element was indicated as a key nutrient for microalgae growth and it was concluded that 0.03 ± 0.02 mM was adequate for optimal growth.

4.2.1.4. Micractinuium pusillum

Micractinium pusillum was grown in a cheese factory anaerobic digestate at 20 ºC and proven to present a satisfactory microalga growth rate. After 4 days, the pH reached 8.5 and the ammonia depletion was complete; although, according to the high pH, it

could be due to the stripping of ammonia or bacterial activity. P-PO_4^{3-} removal reached 33% and the biomass yield was 137 ± 21 mg dry wt/L·d. Moreover, it was observed that the presence of suspended organic matter caused cell clogging and the adhesion of *M. pusillum* to the walls of the culture vessels (Blier et al., 1995).

4.2.2 Cyanobacteria

4.2.2.1. Phormidium bohneri

De la Noüe et al. (1989) also studied the growth of *Phormidium bohneri.* The nitrogen toxic effect for *P. bohneri* was observed at 3.2 mM N-NH_4^+, which indicated that *P. bohneri* presented a higher nitrogen resistance than other common cyanobacteria. Moreover, an increase in temperature (from 10 ºC to 35 ºC) produced an increase in biomass production. It was observed that a concentration of 0.1 – 0.5 mg Cu^{2+}/L showed a toxic effect on *P. bohneri.* 75% of COD removal from the anaerobic digestate was achieved. The higher concentration of *P. bohneri* (32 mg dry wt/L·d) was reached with a 2% swine manure dilution at 20 ºC.

When *P. bohneri* was cultivated in a cheese factory anaerobic digestate at 20 ºC, a rapid increase in pH was observed after 4 days (from 8.4 to 10.9). No significant amount of NH_4^+ was observed after the process; although, according to the high pH, it could be due to the stripping of ammonia or bacterial activity. P-PO_4^{3-}

removal reached 69% with a biomass yield of 329 ± 24 mg dry wt/L·d (Blier et al., 1995).

4.2.2.2. Spirulina maxima

In an early study, *Spirulina maxima* were observed to need a high concentration of bicarbonate ions for optimal growth (Olguin et al., 1994). When it was cultivated in swine manure anaerobic digestate diluted with seawater, an increase in the microalga growth rate was observed with CO_2 supplementation. After 15 days the anaerobic digestate presented a complete $N\text{-}NH_4^+$ reduction, phosphate removal of 99.3%, nitrogen depletion of 76% and a reduction in volatile solids of 28%.

5. Conclusions

Microalgae are renowned as a powerful biotechnology platform for the production of a wide range of value-added products. These include biofuels, animal and aquaculture feeds as well as high-value commercial products such as pigments, polysaccharides, bio-plastics and other organic compounds. Microalgae have also been proposed for a biorefinery model where multiple compounds can be produced simultaneously from harvested microalgal biomass grown in wastewaters and in anaerobic digestion digestates. The growth of the biomass in industrial wastewater and/ or anaerobic digestates has been proven to be a feasible alternative to synthetic mediums.

Regarding the anaerobic digestion of microalgae and cyanobacteria biomass, co-digestion allows to improve the low C/N ratio of microalgae and cyanobacterias, to balance nutrients and to avoid possible inhibitions in many cases. Furthermore, the produced digestate after the anaerobic digestion process presented better stability when a high carbon biomass is co-digested with microalgae or cyanobacteria biomass.

However, the wide variety of microalgae and cyanobacteria as well as the different types of high carbon biomass make it difficult to ascertain a general assessment about the enhancement of methane production when these two biomasses are co-digested. In this respect, it seems that the use of microalgae/bacteria consortium could reduce drawbacks from working with pure species by favouring positive synergetic effects. Further studies will be needed in order to obtain a proper mixture culture.

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Chapter 3

Assessment of two-phase olive mill solid waste and microalgae co-digestion to improve methane production and process kinetics

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Abstract

Olive mill solid waste (OMSW) is a pollutant waste coming from olive oil elaboration by the two-phase centrifugation system. OMSW has a high organic matter content and unbalanced carbon to nitrogen (C/N) ratio, 31/1, which avoids obtaining high methane yields in the anaerobic digestion of this waste. In the present study a microalgae, *Dunaliella salina*, was employed as co-substrate for the OMSW anaerobic digestion in order to decrease the C/N ratio and increase its biodegradability. Different co-digestion mixtures (C/N ratios) were studied. The increase of *D. salina* from 25% to 50% in the co-digestion mixture clearly increased the biodegradability of the sole substrates. The highest biodegradability was found for the co-digestion mixture 50% OMSW-50% *D. salina*. Nevertheless, the maximum methane production, 330 mL CH_4/g VS_{added}, and the highest methane production rate were obtained for the co-digestion mixture 75% OMSW-25% *D. salina*, keeping a C/N ratio near to 26.7/1.

1. Introduction

Over 2.9 million tonnes of virgin olive oil are produced annually worldwide, of which 2.4-2.6 million tonnes are produced in the European Union (IOOC 2009). Over the past decade, Spain has produced between 1.412.000 tonnes (2003/2004 season) and 1.028.000 tonnes (2008/2009 season) of olive oil, which meant 57.7% and 53% of European production (IOOC 2009). Taking into account that oil is only c.a. 20 % (w/w) of the olive, the high quantities of waste produced in the olive oil mills makes sustainable treatments necessary. 98 % of Spanish olive oil mills use the two-phase centrifugation system for olive oil extraction. The main waste produced in this system is the olive mill solid waste (OMSW). The current treatment of OMSW is based on the extraction of the residual olive oil and further combustion. This treatment is not sustainable because of the high water content of the OMSW (Azbar et al., 2004; Celma et al., 2008). There are several experimental treatments for OMSW, such as feedstock for animals, source of pharmaceutical compounds or fertilizer (Martín et al. 2003; Ramos-Cormenzana & Monteoliva-Sánchez 2000; Sierra et al. 2000). An extremely low quantity of OMSW is used in these treatments, so none could be used as an integral treatment for this problematic waste.

Anaerobic digestion of OMSW is a promising technology. Biomethane production between 200-300 mL CH_4/g COD removed and stable continuous reactor operation with organic loading rates

up to 9.2 g COD/(L·d) have been already shown (Rincón et al., 2007). However, organic loading rates higher than 9.2 g COD/(L·d) resulted in a considerable process instability and inhibition. The high content in complex compounds, e. g. cellulose, hemicellulose and phenolic compounds present in the OMSW were most likely responsible for such inhibition (Rincón et al., 2007). In addition, an unbalanced carbon to nitrogen (C/N) ratio 31/1 found in OMSW most likely also avoids a maximal biogas production from this waste.

Co-digestion is proposed in the present study as an approach to dilute complex compounds and balance the C/N ratio. The benefits of co-digestion lie in balancing the C/N ratio in the co-substrate mixture, as well as macro and micronutrients, pH, inhibitors/toxic compounds and dry matter (Hartmann & Ahring, 2005). Low levels of nitrogen, i.e. high C/N ratio, are characterized by a low pH substrate, poor buffering capacity, and the possibility of high volatile fatty acid (VFA) accumulation in the digestion process (Banks & Humphreys, 1998). Low C/N ratios contain relatively high concentrations of ammonia, exceeding that necessary for microbial growth and probably inhibiting anaerobic digestion (González-Fernandez et al., 2011; Yen & Brune, 2007). Several authors have indicated optimum C/N ratio in anaerobic digesters between 20:1 and 30:1 (Habiba et al., 2009; Yen & Brune, 2007).

Anaerobic co-digestion of organic wastes is increasingly being applied for simultaneously treatment of several agro-industrial

solid wastes. Moreover, co-digestion may contribute to a more efficient use of anaerobic reactors and cost-sharing by processing different waste streams in a single equipment (Dareioti et al., 2009).

Microalgae, the common denomination for a broad group of photosynthetic prokaryotes and eukaryotes, are characterized for an efficient conversion of the solar energy to biomass. They are a promising feedstock of biomass for the production of biogas considering both their biomass as energy source and their advantages over traditional land-based energy crops (Salerno et al., 2009). However, microalgae have a very low C/N ratio, which hinders and inhibits a further anaerobic digestion. Ammonia toxicity and recalcitrant cell walls are commonly cited causes of these low methane yields (Sialve et al., 2009). Ammonia toxicity might be counteracted by co-digesting microalgae with high-carbon wastes (Salerno et al., 2009). Yen and Brune (2007) doubled methane production of algal biomass by co-digesting it with waste paper compared with algal biomass alone, with optimum C/N ratio between 20 and 25 (Yen and Brune, 2007). It has been also reported that co-digestion of algae *Spirulina platensis* with WAS improved volatile solids reduction and dewaterability of the digestate compared to WAS alone (Yuan et al., 2012). The same authors reported that co-digestion of algae *Chlorella sp*. with WAS improved volatile solids reduction as well, however, *Chlorella sp.* had a slight negative effect on dewaterability of the digestate

compared to WAS alone (Yuan et al., 2012). Algae biomass residue has also been co-digested with lipid-rich Fat, Oil, and Grease waste (FOG) to evaluate the effect on methane yield (Park and Li, 2012). Co-digestion of algae biomass residue and FOG, each at 50% of the loading, allowed for organic loading rates up to 3 g VS/(L·d), resulting in a specific methane yield of 0.54 L CH_4/(g VS·d) and a volumetric reactor productivity of 1.62 L CH_4/(L·d). Lipids were the key contributor to methane yields, accounting for 68-83% of the total produced methane (Park and Li, 2012).

Dunaliella genus is probably the most halotolerant eukaryotic organisms known, showing a remarkable degree of adaptation to a variety of salt concentrations from 0.2% to salt saturation (Kaçka, A. and Dönmez, 2008). The ability to grow at very high salt concentrations has made these microalgae an attractive candidate for industrial oil transformation which presents a high range of salinity. It could be possible to grow it up in e.g. brine table olives, reducing the need of fresh water and underlined the necessity for very low-cost culture systems. *Dunaliella salina* lacks of a rigid cell wall (Avron and Ben-Amotz, 1992) which most likely would help to the anaerobic digestion process.

The aim of this study was to investigate the possibility of improving methane yield from anaerobic digestion of OMSW in co-digestion with a specific microalga, *D. salina*, based on an optimized C/N ratio. Different co-digestion mixtures were tested in biochemical methane potential (BMP) tests. The influence of the

percentage of each co-substrate on the kinetics of the anaerobic process and ultimate methane yield were also evaluated.

2. Materials and methods

2.1 Two-phase olive mill solid waste

The two-phase OMSW used in the experiments was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). Some of the characteristics of the OMSW used in the experiments are detailed in Table 1. Before to be used, the OMSW was sieved through a 2 mm mesh for removing olive stone pieces.

2.2 Dunaliella salina

Dunaliella salina was provided as a lyophilised by Huelva University, Huelva (Spain). The main characteristics of the *D. salina* used are shown in Table 1.

Table 1. Characteristics of the OMSW and *Dunaliella salina* used in the experiments. Where TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, SCOD: soluble chemical oxygen demand, TKN: Total Kjeldahl nitrogen, TA: total alkalinity, nd: not determined.

Parameters	Values OMSW*	Values *D. salina***
TS (g/kg)	272.2 ± 1.7	908.0 ± 7.3
VS (g/kg)	234.6 ± 2.5	435.8 ± 4.1
COD (g O_2/kg)	331.1 ± 0.7	272 ± 8
SCOD (g O_2/kg)	143.4 ± 3.2	nd
TKN (g/kg)	nd	8.4 ± 0.4
pH	4.9 ± 0.2	8.19±0.1 (1:20)***
TA (g $CaCO_3$/kg)	2.5 ± 0.0	nd

*Concentrations expressed as: weight/weight of wet sample. **Concentrations expressed as: weight/weight of lyophilised sample. *** (w:v) using distillate water.

2.3 Anaerobic sludge

The anaerobic sludge used as inoculum in the BMP tests was obtained from an industrial upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater in Sevilla (Spain). This inoculum was selected due to its high methanogenic activity proven in previous experiments (Rincón et al., 2013). The main characteristics of the inoculum used were: pH: 7.05, total Kjeldahl

nitrogen (TKN): 0.5±0.4 g TKN/kg, total solids (TS): 68.7±0.7 g/kg and volatile solids (VS): 24.7±1.8 g/kg.

2.4 Experimental setup

Different combinations OMSW/*D. salina* were tested: 100% OMSW; 75% OMSW-25% *D. salina*; 50% OMSW-50% *D. salina*; 25% OMSW- 75 % *D. salina* and 100% *D. salina* corresponding to C/N ratios of: 31.4, 26.7, 22.0, 17.3 and 12.6, respectively.

The biochemical methane potential (BMP) tests were carried out in a multi-batch reactor system; effective volume of reactors was 250 mL. They were continuously agitated by magnetic bars at 500 rpm and placed in a thermostatic water bath at mesophilic temperature (35±2 ºC).

The inoculum to substrate ratio was 2 (VS basis). For each reactor containing 239 mL of inoculum, the amount of substrate needed to give the required inoculum to substrate ratio was added together with 239 µL of trace element solution.

The composition of the trace elements solution was: $FeCl_2 \cdot 4H_2O$, 2000 mg/L; $CoCl_2 \cdot 6H_2O$, 2000 mg/L; $MnCl_2 \cdot 4H_2O$, 500 mg/L; $AlCl_3 \cdot 6H_2O$, 90 mg/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 mg/L; H_3BO_3, 50 mg/L; $ZnCl_2$, 50 mg/L; $CuCl_2 \cdot 2H_2O$, 38 mg/L, $NiCl_2 \cdot 6H_2O$, 50 mg/L, $Na_2SeO_3 \cdot 5H_2O$ 194 mg/L and EDTA 1000 mg/L. Two reactors with inoculum and trace elements solution but without substrate addition were used as controls.

The reactors were sealed and the headspace of each flask was

flushed with nitrogen at the beginning of the assay. The produced biogas was passed through 3N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. The anaerobic digestion experiments were run for a period of c.a. 25 days until the accumulated gas production remained essentially unchanged, i.e. on the last day production was lower than 2% of the accumulated methane produced. Each experiment was carried out in duplicate.

2.5 Analytical methods

All analyses were performed according to the Standard Methods of APHA (APHA, 1998). The following parameters were measured: total chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), total solids (TS), volatile solids (VS), total alkalinity (TA), pH, Total Kjeldahl Nitrogen (TKN) and elemental C and N.

TS and VS were determined according to the standard methods 2540B and 2540E (APHA, 1998), respectively; COD was determined by the method described by Raposo et al. (2008), while SCOD was determined using the closed digestion and the colorimetric standard method 5220D (APHA, 1998). pH was analysed using a pH-meter model Crison 20 Basic. TA was determined by pH titration to 4.3 (APHA, 1998). TKN was determined using a method based on the 4500-N_{org} B of Standard Methods (APHA, 1998). C and N were determined through an Elemental Analyser LECO CHNS-932 (Leco Corporation, St

Joseph, MI, EEUU).

3. Results and discussion

3.1 Influence of co-digestion on biochemical methane potential

Figure 1 shows the variation of the methane yield obtained (mL CH_4/g VS added) against digestion time (days) for the BMP tests carried out with 100% OMSW, 100% *D. salina*, and with the co-digestion mixtures 75% OMSW-25% *D. salina*, 50% OMSW-50% *D. salina*, 25% OMSW-75% *D. salina.*

The experimental methane yields observed for each co-digestion mixture (Figure 1) were compared to a calculated methane yields based on the OMSW and *D. salina* methane yields separately according to the equation 1:

$$\text{Calculated methane yield (mL } CH_4\text{/g } VS_{added}) = \text{\% OMSW} \cdot (321) + \text{\% } D.\ salina \cdot (63) \quad (1)$$

Where 321 and 63 are the experimental methane yields (mL CH_4/g VS added) obtained from 100% OMSW and 100% *D. salina*, respectively. % OMSW and % *D. salina* are the percentages of OMSW and *D. salina* in each co-digestion mixture. The experimental methane yield values obtained for all experiments

performed and the calculated ones are summarized in Table 2.

Experimental BMP values were higher than the calculated methane yield from eq. 1 in each of the co-digestion mixture tested (Table 2). 28% for co-digestion mixture 75 % OMSW-25 % *D. salina*, 48% for co-digestion mixture 50 % OMSW-50 % *D. salina* and 3% for co-digestion mixture 25 % OMSW-75 % *D. salina*. According to the increase of BMP values, the biodegradability of the co-digestion mixtures were as well much higher than the biodegradability of the sole substrates (Table 2). The biomethane potential of the OMSW was found very low, as only 56.9% of the available COD is converted to methane. The biomethane potential of the *D. salina* was found very low as well, 25% of the available COD was converted to methane (Table 2). The co-digestion mixture 50 % OMSW-50 % *D. salina* had a biodegradability of 73.2 and the co-digestion mixture 75% OMSW-25% D. *salina* of 71.5. Synergy effect of the OMSW and *D. salina* co-digestion was clearly shown with these results.

Table 2. Calculated methane yield values obtained from eq. 1, experimental data obtained through BMP test and biodegradability of the different co-digestion mixtures

C/N ratio	OMSW	*D. salina*	Calculated	Experimental	Methane yield improvement	Biodegradability (COD-CH_4 / CODadded)
	(%)	(%)	(mL CH_4/g VS added)	(mL CH_4/g VS added)	(%)	(%)
31.4	100	0	321	321	0	56.9
26.7	75	25	257	330	28	71.5
22.0	50	50	192	285	48	73.2
17.3	25	75	128	132	3	45.8
12.6	0	100	63	63	0	24.7

D.salina= Dunaliella salina.

Although the co-digestion mixture 50 % OMSW-50 % *D. salina* increased 48% the methane yield with respect to its calculated value and had a biodegradability of 73.2% (Table 2), the co-digestion mixture 75% OMSW-25% *D. salina* was the combination with the highest methane yield, i.e. 330 mL CH_4/g VS added. 75% OMSW-25% *D. salina* co-digestion mixture corresponded to a C/N ratio of 26.7/1, an intermediate value between 20/1 and 30/1 described as optimum range in literature (Habiba et al., 2009). Furthermore, the methane yield value obtained for the mixture 75% OMSW-25% *D. salina* was 15.8% and 150% higher than those achieved for the mixtures 50% OMSW-50% *D. salina* and 25% OMSW-75% *D. salina*, respectively.

The unbalanced C/N ratios of the algal biomass have been reported as an important limitation factor to anaerobic digestion processes. It has been reported that the addition of waste paper (50% based on VS) in algal biomass feedstock to maintain an optimum C/N ratio (20-30) double the methane production rate (Yen and Brune, 2007). The latter authors claimed that the stimulation of the cellulose activity by the presence of the waste paper had a positive effect on the anaerobic digestion of algal cell walls (Yen and Brune, 2007). Co-digesting studies with a mixture of algae, effluent from canning industry and protein-extracted algae also demonstrated that the optimum C/N ratio to achieve a maximum methane production was found between 20 and 30 (Chen, 1987). C/N ratios lower than 20/1 lead to potential

inhibition due to the presence of free ammonia whereas C/N ratios higher than 30/1 may lead to potential nitrogen limitations (Sialve et al., 2009).

The lowest methane yields obtained in the present study corresponded to the 100% D.*salina* and for the co-digestion mixture 25% OMSW-75% *D. salina*. González-Fernández et al., (2011) reported in the co-digestion of microalgal biomass constituted by *Chlorella vulgaris, Scenedesmus obliquus* and swine manure that the methane yield decreased from 221 to 143 mL CH_4/g COD when the percentage of total COD provided by algal biomass increased from 14.6% to 85.4%, the lowest value was achieved for the digestion of algal biomass as a sole substrate (128 mL CH_4/g COD). This result was attributed to the hemicellulosic cell wall of these two species of microalgae, which present a high resistance to bacterial degradation (González-Fernández et al., 2011).

It has been also reported the anaerobic co-digestion of cattle excreta and OMSW (Goberna et al., 2010). The mesophilic co-digestion at a 3:1 ratio rendered 1096 mL biogas/(L sludge d), value 33% higher than that of excreta alone. The methane yield resulting from the co-digestion was 179 mL CH_4/g VS added, of which 42% was attributed to OMSW (Goberna et al., 2010). This methane yield value was considerably lower than those obtained in the present work for the co-digestion mixtures 75% OMSW-25%

D. salina (330 mL CH_4/g VS added) and 50% OMSW-50% *D. salina* (285 mL CH_4/g VS added).

3.2 Influence of co-digestion on process kinetics

3.2.1 Kinetic models of methane production

Two different periods were clearly differentiated in the evolution of methane production with time for the digestion of 100% OMSW and for the co-digestion mixtures (Figure 1).

A first stage, during the first 5 days of operation, followed by an intermediate adaptation period or lag stage, and finally, a second phase, in which the methane production rate increased gradually to become almost zero at the 15-20 days of digestion were observed (Figure 1). A similar trend was observed previously by Rincón et al (2013) with OMSW as substrate. Only the first stage was observed for the digestion of 100% *D. salina*. Therefore, OMSW digestion is clearly the reason for such a two stages methane production profile. In order to simulate the two stages observed, two different models were selected and used as previously by Rincón et al. (2013). A first-order exponential model for the first stage which is commonly applicable to easily biodegradable substrates (Li et al., 2012) and a second sigmoidal or logistic model for the lag and second stage with its three characteristic phases, i.e. lag, exponential increase and final stabilization step (Donoso-Bravo et al., 2010).

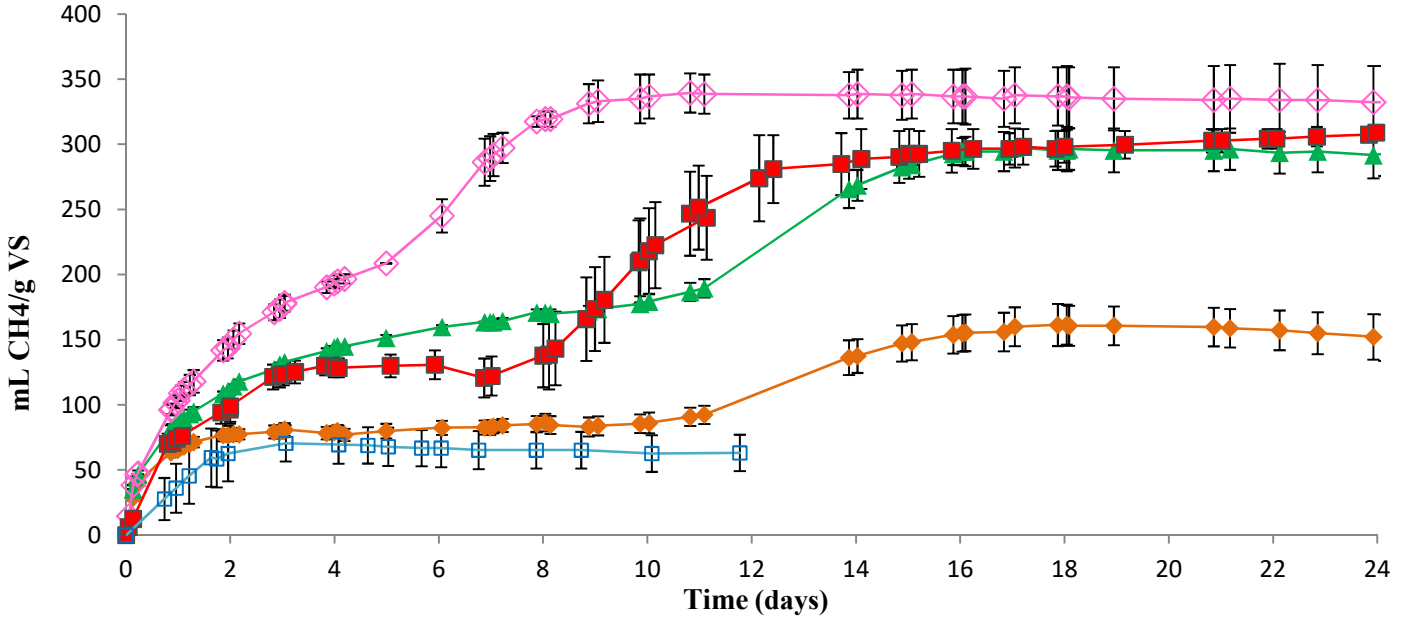

Figure 1. Biochemical methane potential (mL CH_4/g VS added) of 100% OMSW (■), 100% *Dunaliella salina* (□) and different co-digestion mixtures tested: 75% OMSW-25% *D. salina* (◊); 50% OMSW-50% *D. salina* (▲) and 25% OMSW- 75 % *D. salina* (♦).

3.2.2 First-order exponential model

The first-order exponential model is given by the equation 2:

$$B_1 = B_{max} \cdot [1 - \exp(k \cdot t)] \quad (2)$$

where: B_1 (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, k is the specific rate constant or apparent kinetic constant ($days^{-1}$) and t (days) is the time.

This model was applied for the first experimental stage of methane production or exponential step (first 5 days of digestion) for digestion of 100% OMSW and for co-digestion mixtures. Moreover, this model was the only one applied in the case of digestion of 100% *D. salina*.

The adjustment by non-linear regression of the pairs of experimental data (B_1, t) using the Sigmaplot software (version 11.0) allowed the calculation of the parameters k and B_{max} for this first stage of methane production (Table 3). The high values of the R^2 and the low values of the standard error of estimate for all cases tested demonstrate the goodness of the fit of experimental data to the model proposed for this first exponential stage (Table 3, Figure 2).

k values obtained for the first stage of digestion were very

similar for digestion of 100% OMSW and for co-digestion mixtures 75% OMSW-25% D. salina and 50% OMSW-50% D. salina, with values ranging between 0.69±0.04 and 0.78±0.04 days-1 (Table 3). The lowest k value, i.e. 0.49 days-1, was achieved for 100% D. salina digestion. The low C/N ratio of the microalga alone, i.e. 12.6, is most likely the reason for such a low k value. The highest k value in the first stage was obtained for the co-digestion mixture 25% OMSW-75% D. salina, i.e. 2.2 days-1 (Table 3). The increase of OMSW to the co-digestion mixture resulted in lower k values but higher Bmax than the co-digestion mixture 25% OMSW-75% D. salina (Table 3). The increase in the C/N ratio improved the total methane production, however, had a negative effect on the initial degradation rate. This negative effect might be attributed to an increasing concentration of complex compounds in the co-digestion mixture coming from the OMSW (Rincón et al., 2007).

Table 3. Kinetic parameters obtained from the exponential model in the BMP tests of digestion of 100% *D. salina*, 100% OMSW and for co-digestion mixtures 75% OMSW-25% *D. salina*, 50% OMSW-50% *D. salina* and 25% OMSW-75% *D. Salina.*

Substrate	B_{max} (mL CH_4/g VS_{added})	k ($days^{-1}$)	R^2	**S.E.E.**
100% *D. salina*	62±4	0.49±0.08	0.9558	3.99
25% OMSW-75% *D. salina*	76.7±0.8	2.2±0.1	0.969	3.64
50% OMSW – 50% *D.*	161±3	0.69±0.04	0.958	9.72
75% OMSW – 25% *D.*	198±5	0.75±0.04	0.983	7.59
100% OMSW	133±2	0.78±0.04	0.991	4.26

B_{max} is the ultimate methane production, k is the specific rate constant or apparent kinetic constant. Parameters from the nonlinear regression fit: R^2: coefficient of determination; S.E.E.: Standard Error of Estimate.

3.2.3 Sigmoidal or logistic model

For the second stage of methane production, i.e. between the 5th and last day of the operating period: 24-25th day, the following logistic model (eq. 3) was used to estimate process performance (Donoso-Bravo et al., 2012; Li et al., 2012; Rincón et al., 2013):

$$B_2 = B_0 + P/[1 + \exp(-4 \cdot R_m \cdot (t - \lambda)/(P + 2))] \quad (3)$$

where: B_2 is the cumulative methane production during the second stage (mL CH_4/g VS_{added}), B_0 is the cumulative methane production at the start-up of the second stage (mL CH_4/g VS_{added}) and should approximately coincide with the value of B_{max} obtained at the end of the first stage, P is the maximum methane production obtained in the second stage (mL CH_4/g VS_{added}), R_m is the maximum methane production rate (mL CH_4/(g VS_{added} · d)) and λ is the lag time (days).

The logistic model assumes the rate of methane production to be proportional to microbial activity (Altas, 2009). The logistic model fairly fits the methane production during the second stage (5-25 days): an initial lag period followed by an exponential increase and a final stabilization at a maximal production level (Figure 2, Table 4). This model has been previously used for estimating the methane production in batch anaerobic digestion experiments of

different substrates such as landfill leachate, herbaceous grass materials, sewage sludge, etc. (Altas, 2009; Donoso-Bravo et al., 2010; Li et al., 2012; Pommier et al., 2006). Table 4 summarizes the kinetic parameters obtained from the logistic model in the BMP tests of the different mixtures tested. The highest estimated lag periods for the logistic model were found for the co-digestion mixtures 25% OMSW-75% *D. salina* and 50% OMSW-50%.

The increase of *D. salina* in the co-digestion mixture clearly increased the lag period for the second stage. The high estimated lag time found for mixtures containing the highest proportion of microalgae may be attributed to the high protein content of the algal biomass, which leads to a high ammonium release, thus inhibiting the anaerobic microorganisms (Sialve et al., 2009). The lowest estimated lag period corresponded to the mixture 75% OMSW-25% *D. salina*, i.e. 6.4 days (Table 4). The estimated lag period for 100% OMSW was 9.9 days (Table 4). This observation indicates that, although the increase of *D. salina* over 50% promotes inhibition to the microbial community, an adequate addition would definitely increase the methane production*D.salina*.

Table 4. Parameters obtained from the logistic model in the BMP tests of 100% OMSW and for co-digestion mixtures 75% OMSW-25% *D. salina*, 50% OMSW-50% *D. salina* and 25% OMSW-75% *D. salina.*

Substrate	B_0 (mL CH_4/g VS_{added})	P (mL CH_4/g VS_{added})	R_m (mL CH_4/(g VS·d))	λ (days)	R^2	S.E.E.
25% OMSW-75% *D. salina*	77.5±0.7	72.4±0.8	18.8	12.8±0.	0.999	1.00
50% OMSW-50% *D. salina*	165±1	132±1	30.1	12.6±0.	0.999	1.22
75% OMSW-25% *D. salina*	188±2	149±2	48.1	6.4±0.1	0.998	1.51
100% OMSW	118±10	181±11	38.4	9.9±0.2	0.995	4.98

B_0 is the cumulative methane production at the start-up of the second stage (mL CH_4/g VS_{added}), P is the maximum methane production obtained in the second stage (mL CH_4/g VS_{added}), R_m is the maximum methane production rate (mL CH_4/g VS_{added}·d) and λ is the lag time (days). R^2: coefficient of determination; S.E.E.: Standard Error of Estimate.

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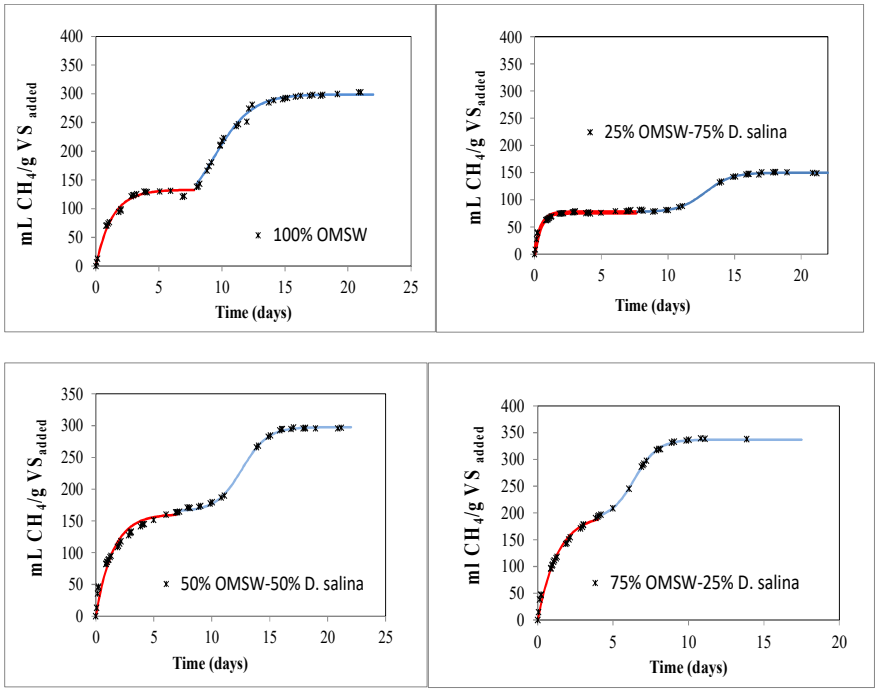

Figure 2. Cumulative methane yield expressed as mL CH_4/g VS added (*), first stage exponential model (**---**) and (---) second stage logistic model for 100% OMSW and the different co-digestion mixtures studied: 75% OMSW-25% *D. salina*, 50% OMSW-50% *D. salina,* and 25% OMSW- 75 % *D. salina*.

The value of R_m for the mixture 75% OMSW-25% *D. salina* was 60% and 155% higher than those obtained for the mixtures 50% OMSW-50% *D. salina* and 25% OMSW-75% *D. salina*

respectively. To be specific it was observed a decrease in the maximum methane production rate (R_m) of this stage from 48.1 to 18.8 mL CH_4/(g VS·d) when the percentage of *D. salina* in the mixture increased from 25% to 75%.

The ammonia release during the co-digestion of increased concentrations of microalgae could explain the poorer digestion performance and slower kinetics when the percentages of microalgae in the co-digestion mixture were increasing (González-Fernández et al., 2011; Sialve et al. 2009).

The first derived of B_2 with respect to the digestion time gives the evolution of the methane production rate, which maximum corresponds to R_m, with time during the second stage (Figure 3). The degradation rate of the co-digestion mixture 75% OMSW-25% *D. salina* was the fastest of the three conditions tested, achieving the R_m, i.e. 48.1 mL CH_4/(g VS·day), after 6.3 days of digestion time. The time to achieve R_m was much higher for the other tests than for the 75% OMSW-25% *D. salina.* 50% OMSW-50% *D. salina* achieved R_m, i.e. 30.1 mL CH_4/(g VS·day), after 12.5 days of digestion. 25% OMSW-75% *D. salina* achieved R_m, i.e. 18.8 mL CH_4/(g VS·day), after 12.5 days of digestion as well. 100% OMSW achieved R_m, i.e. 38.4 mL CH_4/(g VS·day), after 9.4 days. The co-digestion mixture 75% OMSW-25% *D. salina* was the fastest and the one that produces the highest amount of biomethane among all the co-digestion mixtures tested.

3.3 Influence of co-digestion on the olive mill sustainability

The use of *D. salina,* together with OMSW (75% OMSW-25% *D. salina*) allows obtaining higher methane yields from OMSW than using OMSW alone. The energy obtained in the digestion process could be used to keep the mesophilic operating temperature (35 ºC) of the anaerobic reactor and even in the own olive oil elaboration. Moreover, the effluents obtained in the anaerobic digester might be used as fertilizer in olive trees fields and as nutrient source for new microalgae cultivation. All this improves the whole sustainability of the olive oil elaboration system by means of close loops. Furthermore, the use of a saline microalga as *D. salina* would allow the use of sea water or salted concentrated industrial streams, e.g. olive brine, as growth media, decreasing the need of fresh water.

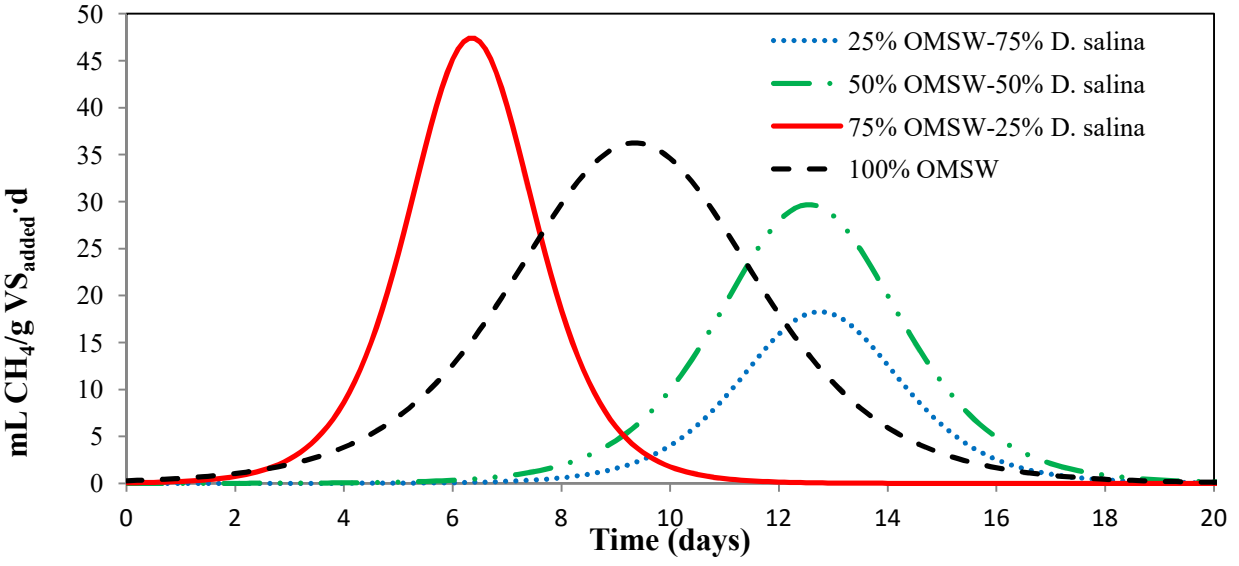

Figure 3. Methane production rate, expressed as mL CH_4/(gVS day) obtained during the second stage of the BMP test carried out with the 100% OMSW and the different co-digestion mixtures: 75% OMSW-25% *D. salina*, 50% OMSW-50% *D. salina,* and 25% OMSW- 75 % *D. salina*.

Conclusions

Anaerobic co-digestion of OMSW and *D. salina* with a mixture of 75%-25% respectively, keeping a C/N ratio of 26.7, increased the methane yield and the methane production rate compared to anaerobic digestion of 100% OMSW, 100% *D. salina* and other co-digestion mixture percentages. Nevertheless, anaerobic co-digestion of 50% OMSW-50% *D. salina* had a higher synergic effect than the other co-digestion mixtures studied. Although the 50% co-digestion mixture increased biodegradability of OMSW and *D. salina*, the 75% OMSW-25% *D. salina* co-digestion mixture would allow operating with smaller anaerobic digesters or lower retention times and with a still high biodegradability of OMSW.

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Chapter 4

Anaerobic co-digestion of olive mill solid waste and microalga *Scenedesmus quadricauda*: Effect of different carbon to nitrogen ratios on process performance and kinetics

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Abstract

Anaerobic digestion (AD) is one of the most efficient processes for treating agri-food waste in order to obtain renewable energy. Olive mill solid waste (OMSW) is the main residue from the two-phase olive oil manufacturing process; it has a high organic content and high C/N ratio, which hinders its AD, giving low methane yield. In the present study, a microalga, *Scenedesmus quadricauda* (*S. quadricauda*), was used as co-substrate for the AD of OMSW to compensate for its nitrogen deficiency. The robustness and the high growth rate of *S. quadricauda* make this microalga a potential source of nitrogen to co-digest with carbon-rich substrates. Different co-digestion mixtures of OMSW-microalgae and the single substrate were tested. For all co-digestion mixtures, the alkalinity value at the end of the experiment remained below 4889 ± 245 mg $CaCO_3$/L and pH in the range of 7.50-7.67 indicating stability and good process performance. The results showed the highest methane yield (461 mL CH_4 STP/g VS added) for the co-digestion mixture 75% OMSW-25% *S. quadricauda* (C/N = 25.3), which was 104 and 23% higher than that obtained from the single microalga (C/N = 5.6) and OMSW (C/N = 31.9), respectively. No ammonia inhibition was detected despite the high protein content of the microalgae. The transference function model allowed for adequately fitting the experimental results of methane production

with time in the anaerobic experiments. The highest maximum methane production rate, Rm, among the different co-digestion mixtures assayed was obtained for the mixture 75% OMSW-25% *S. quadricauda* with a value of 89 mL CH_4/(g VS d).

1. Introduction

The Mediterranean culture has always been linked to the consumption of olive oil, and countries in Southern Europe, North Africa and the Middle East are its main producers. Within these countries, Spain is worth mentioning as the main producer and exporter of olive oil worldwide. The main problem of olive oil production is the generation of highly-polluting by-products which cause several environmental problems (Rincón et al., 2008).

At the beginning of the 90s, oil mills began to establish a new system for the extraction of olive oil by two-phase centrifugation. The two-phase olive oil system contributed to an improvement in the quality of the product and a marked decrease in the use of water during the process. Currently, more than 90% of Spanish olive oil industries have implemented the more sustainable two-phase system, even though 800 kg of the main waste are still produced for each tonne of processed olive (Fernández Rodríguez et al. 2014). The main by-product generated from this system is the olive mill solid waste (OMSW). The large amount of OMSW produced every year, generates several environmental problems (Khatib et al. 2009); not only because of the large amount generated, but also because of its characteristics and chemical composition. OMSW is a lignocellulosic byproduct characterized by its high organic content and phenolic compounds. There are several experimental

treatments for OMSW, which have the potential to make it re-usable as fertiliser and water for irrigation or to obtain valuable materials from it such as, proteins, minerals and polysaccharides (Pantziaros et al. 2018; Sousa et al. 2018). However, the reuse of OMSW is restricted by the abundance of complex compounds it contains and its elevated polluting load. Anaerobic digestion (AD) is the most viable treatment option because of its advantages related to energy production, high chemical mineralization and low sludge production (Rincón et al. 2008).

AD of organic waste has been applied extensively for decades to produce biogas and several studies of AD on the OMSW have been reported (de la Lama et al. 2017; Gunay and Karadag 2015). However, using OMSW as the sole substrate is not recommended due to its nutritional imbalance (Fernández Rodríguez et al. 2014). OMSW is a recalcitrant substrate that has a low methane potential due to its lignocellulosic content (Maamir et al. 2017). Hydrolysis of lignocellulosic wastes, such as OMSW, is often the rate-limiting step in anaerobic processes (Wang et al., 2006). There are several methods to enhance the destruction of the biopolymers and complex substances in order to increase biogas production. Anaerobic co-digestion of two substrates is proposed in the present study to maximize biogas production. The benefits of co-digestion lie in balancing the C/N ratio in the co-substrate mixture, as well as macro and micronutrients, pH, inhibitors/toxic compounds and also

accelerating the hydrolysis process (Barua et al. 2019). As benefits of co-digestion Ajeej et al. (2015) reported an increased activity of methanogenic bacteria, a decrease in AD inhibition by ammonium and even an increase in cellulose activity when carbon-rich materials were added.

The addition of nitrogen-rich biomass, such as microalgae, could balance the C/N ratio, thus accelerating the hydrolytic phase and providing a constant concentration of nitrogen to the substrate. Some studies have been carried out with the aim of investigating the best performance of anaerobic co-digestion based on C/N ratio optimization (Fernández-Rodríguez et al. 2014; Ambarsari et al. 2018; Xie et al. 2018; Nguyen et al. 2018). For instance, Fernández-Rodríguez et al. (2014) reported a maximum methane yield when a mixture between OMSW and the microalga *Dunaliella salina* had a C/N close to 26.7/1.

The feasibility of using algal biomass cultivated with wastewater adds more value to these studies due to a combination of nutrient removal from wastewater with CO_2 fixation which provides an economically viable system. On the one hand, there are studies that show the capacity for growing microalgae in wastewater and achieving a maximum COD removal efficiency of 72.3% (Xu et al. 2015). On the other hand, microalgae can be used

as co-substrate in an approach to dilute complex compounds and balance the C/N ratio.

Scenedesmus is one of the most common genera of Chlorophyta, and is characterized by a rigid, sugar cell wall (Takeda 1996). It is one of the most commonly used microalgae due to its plasticity, its potential for rapid growth and ability to grow in different environments. There are numerous studies that support the robustness and fast growth of this genus in wastewater, and its effectiveness in removing a high percentage of nutrients from wastewater treatment plants (Xiao et al. 2011; Wong et al. 2015). There are even studies that characterize *Scenedesmus* as a genus able to bio-accumulate lipids, highly-degradable organic compounds during anaerobic digestion (Rincón et al. 2018).

Therefore, the aim of this study was to evaluate the stability of the system, its energy recovery potential and macronutrient (N) removal from the various co-digestion mixtures of OMSW and *Scenedesmus quadricauda* (*S. quadricauda*) in order to study the potential of the robust and fast growing microalgae *S. quadricauda* as feedstock for co-digestion with OMSW in batch experiments. The influence of the percentage of each co-substrate on the kinetics of the anaerobic process and ultimate methane yield was also evaluated in biochemical methane potential (BMP) tests. BMP is a procedure developed to determine the methane production of a

given organic substrate during its anaerobic decomposition. The BMP assay has proved to be a relatively simple and reliable method to obtain the extent and rate of organic matter conversion to methane. The information provided by BMP is valuable when evaluating potential substrates and for optimizing the design and functioning of an anaerobic digester. Literature related to BMP assays is extensive showing that this test has been used to evaluate a wide variety of substrates (Holliger et al., 2016).

2. Material and methods

2.1 Two-phase olive mill solid waste

The two-phase OMSW used in the experiments was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). Before use, the OMSW was sieved through a 2 mm mesh for removing pieces of olive stone. Some of the characteristics of the OMSW used in the experiments were shown in Table 1.

*2.*2 Microalgal strain and cultivation

The Chlorophyta *S. quadricauda* was grown at 25 ºC in BG11 medium (Rippka et al. 1979). The algal biomass was cultivated during two weeks under 12 : 8 light : dark cycles in an AGP-700-ESP incubator chamber (Rdiber S.A., Barcelona, Spain) with illumination provided by 6 fluorescent lamps (36 W). Periodically microscope observations were carried out to ensure that the algae culture was composed mainly of *Scenedesmus* sp. The cells from the culture were concentrated by centrifugation (5 min at 3.500 rpm). Finally, microalgal biomass was placed in liquid nitrogen (-196 °C) and stored at -80 ºC. Some characteristics of the algal biomass used in the experiments were also shown in Table 1.

*2.*3 Inoculum for anaerobic digestion

The inoculum was obtained from an industrial upflow anaerobic sludge blanket (UASB) reactor which treats brewery wastewater located in Sevilla (Spain). The main characteristics of the inoculum used were summarized in Table 1.

2.4 Experimental procedure

The test was carried out in a multi-batch reactor system. The effective volume of the reactors was 250 mL. The BMP test was performed in triplicate and the reactors were continuously agitated by magnetic bars at 500 rpm and placed in a thermostatic water bath at mesophilic temperature (35±2 ºC).

The inoculum-to-substrate ratio was 2 (VS basis). A solution of trace elements was added to each reactor containing 239 mL of inoculum and the required amount of substrate. The composition of the solution of trace elements was: $FeCl_2 \cdot 4H_2O$, 2000 mg/L; CoCl2·$6H_2O$, 2000 mg/L; MnCl2·$4H_2O$, 500 mg/L; $AlCl_3 \cdot 6H_2O$, 90 mg/L; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 50 mg/L; H_3BO_3, 50 mg/L; $ZnCl_2$, 50 mg/L; $CuCl_2 \cdot 2H_2O$, 38 mg/L, $NiCl_2 \cdot 6H_2O$, 50 mg/L, $Na_2SeO_3 \cdot 5H_2O$ 194 mg/L and EDTA 1000 mg/L. As controls, three 250 mL batch reactors were supplied with the inoculum and trace elements solution but without the addition of substrate. The reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 3N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. The anaerobic digestion experiments were run for a period of c.a. 20 days until the accumulated gas production remained essentially unchanged. The volume of methane produced was corrected at standard temperature

and pressure conditions (STP: 0 ºC, 1 atm). The C/N ratios of five different mixtures were tested, and ranged from 31.9 (100% OMSW) to 5.6 (100% microalga). Intermediate C/N ratios like 12.1 (25% OMSW-75% *S. quadricauda*), 18.7 (50% OMSW-50% *S. quadricauda*) and 25.3 (75% OMSW-25% *S. quadricauda*) were also tested.

Table 1. Characteristics of the olive mill solid waste (OMSW), *Scenedesmus quadricauda* (*S. quadricauda*) and the inoculum used in the experiments. Where TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, SCOD: soluble chemical oxygen demand, TA: total alkalinity, nd: not determined.

Parameters	Values OMSW*	Values *S. quadricauda**	Values Inoculum*
TS (g/kg)	267.2 ± 1.6	99.6 ± 5.8	32.9 ± 0.3
VS (g/kg)	235.1 ± 0.8	94.0 ± 3.6	23.7 ± 0.3
COD (g O_2/kg)	329.8 ± 1.3	75.5 ± 2.8	nd
SCOD (g O_2/kg)	144.4 ± 2.6	nd	nd
TA (g $CaCO_3$/kg)	2.5 ± 0.1	nd	nd
pH	4.8 ± 0.2	nd	6.98 ± 0.0

*Concentrations expressed as: weight/weight of wet sample.

2.5 Analytical methods

All analyses were performed according to the Standard Methods of APHA (APHA 1998). The following parameters were measured: total chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), total solids (TS), volatile solids (VS), total alkalinity (TA), pH, ammonium concentration ($N\text{-}NH_4^+$) and elemental C and N. Soluble parameters were determined after sample centrifugation (Eppendorf, 10000 rpm, 10 min) and filtration (glass fiber filter 47mm). Free ammonia concentration was calculated according to the following formula (Østergaard, 1985):

$$\frac{[NH_3]}{[TNH_3]} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}}\right)^{-1} \quad (1)$$

where $[NH_3]$ is the concentration of free ammonia, $[TNH_3]$ is the total ammonia concentration and T(K) is the temperature measured in degrees kelvin.

TS and VS were determined according to the standard methods 2540B and 2540E (APHA 1998), respectively; COD was determined by the method described by Raposo et al. (2008), while SCOD was determined using the closed digestion and the colorimetric standard method 5220D (APHA 1998). pH was analyzed using a pH-meter model Crison 20 Basic. TA was determined by pH titration to 4.3 (APHA 1998). Ammonium was determined colorimetrically according to the phenol–hypochlorite method based on Standard Methods (APHA 1998). C and N were determined through an Elemental Analyzer LECO CHNS-932 (Leco Corporation, St Joseph, MI, EEUU). Individual volatile fatty acids (VFA) from C2 to C7 including iso-C4, iso-C5 and iso-C6 were analyzed by a Gas Chromatograph (Shimadzu GC 2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol (polyethylene glycol modified by nitroterephthalic acid). Prior to injection, 900 µL of the sample were mixed with 150 µL of H_3PO_4 (1:2 V:V) to adjust the pH to below 2.0 and 150 µL of a crotonic acid solution (2000 mg/L) were added as internal standard. This mixture was centrifuged to remove any solids and transferred to a 1500 µL gas chromatography (GC) vial; the sample injection volume was 1 µL. The temperatures of the injector and detector were maintained at 200 and 250 ºC, respectively, while the column temperature was increased from 120 to 160 ºC at a rate of 10 ºC/min.

2.6 Kinetic study

The Transference Function (TF) model was applied to fit the experimental data of methane production during BMP tests (eq. 2). The transference function *(Reaction curve-type model*) (RC), used mainly for control purposes, considers that any process might be analyzed as a system receiving inputs and generating outputs (Donoso-Bravo et al. 2010). The TF model was successfully applied by several authors for the biomethanization of different organic wastes (Donoso-Bravo et al. 2010; Li et al. 2012; Pinto-Ibieta et al. 2016). The TF model is given by the following expression:

$$B = B_{max} * \left(1 - exp\left[-\frac{R_{max}(t-\gamma)}{B_{max}}\right]\right) \quad (2)$$

where B (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, R_{max} is the maximum methane production rate (mL CH_4/(g VS_{added}*d)), t (d) is the digestion time and γ (d) is the lag time.

Error (%), determination coefficient (R^2) and standard error of estimate were calculated to evaluate the goodness-of-fit and the accuracy of the results. Error was defined as the percentage

difference between the experimental and the predicted or theoretical methane yield coefficient. The kinetic parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 11).

3. Results and discussion

3.1 Stability of the system

Due to the great fragility and slow growth rate of methanogenic microorganisms, it is important that the process conditions be sufficiently stable to guarantee maximum methane production during the AD process. The total alkalinity of the system is a parameter related to the buffer capacity of the medium. Alkalinity is influenced by carbonate, ammonium, phosphate, VFA and sulphide subsystem (Xue et al. 2017). Fannin and Biljetina (1987) stablished values between 2500- 5000 mg $CaCO_3$/L as ideal for the process of anaerobic digestion. At the end of the experiment, the total alkalinity values ranged from 3471±176 mg $CaCO_3$/L to 6132±59 mg $CaCO_3$/L (100% OMSW and 100% *S. quadricauda*, respectively) (Table 2). Intermediate total alkalinity values were observed at the end of the experiment for the different co-digestion mixtures: 4889±245 mg $CaCO_3$/L, 4455±574 mg $CaCO_3$/L and

4804±115 mg $CaCO_3$/L, for the mixture 75% OMSW- 25% *S. quadricauda*, 50% OMSW- 50% *S. quadricauda*, and 25% OMSW- 75% *S. quadricauda*, respectively (Table 2). The AD of 100% *S. quadricauda* was the only one that had total alkalinity values higher than those stablished by Fannin et al.,[23] as the optimal value for the stability of AD. The high nitrogen content of microalgae may explain the increase in total alkalinity due to the increased in ammonia concentration, which might initially prove extra alkalinity (Zhang et al. 2016). When the concentration of the ammonia begins to increase, instability and system failure of the AD process were observed. The adjustment of the C/N ratio during co-digestion can minimize this problem. Volatile fatty acids/ total alkalinity ratios (VFA/TA) is a reliable indicator of process stability in an anaerobic digestion system. When pH values are between 6 and 8, the main chemical equilibrium which controls the alkalinity is carbonic acid- bicarbonate. In order to avoid the acidification of the reactor, the VFA/TA ratio has to be less than 0.3-0.4 (Iza 1995). In fact, all the VFA/TA ratio values showed during anaerobic co-digestion were clearly lower than 0.3. In contrast to this, the value of the VFA/TA ratio during anaerobic digestion of 100% microalga was close to 0.4. Therefore, co-digestion provided balanced nutrients and prevented the acidification of the AD process. Similar results were reported by Li et al. (2016).

Table 2. Chemical composition of the biochemical methane potential (BMP) test effluents.

OMSW (%)	*S. quadricauda* (%)	TA (mg $CaCO_3$/L)	VFA/TA	pH	ammonium (mg/L)	ammonia (mg/L)
100	0	3471±176	0.30	7.78	1224±24	35.9
75	25	4889±245	0.22	7.67	1082±2	24.8
50	50	4455±574	0.25	7.65	902±21	19.7
25	75	4804±115	0.15	7.50	935±25	14.6
0	100	6132±59	0.39	7.72	882±12	22.6

*OMSW: olive mill solid waste, *S.quadricauda: Scenedesmus quadricauda,* TA: Total alkalinity, VFA/TA: volatile acids/total alkalinity ratio.

3.2 Nitrogen

The algal cell wall and ammonium toxicity are the main factors to guarantee the optimal biogas production during the AD of microalgae (González-González et al. 2018). Microalgae have a high nitrogen content (C/N ratio = 5.6) so it was expected that methane production was inhibited by one of its main methanogen inhibitors, free ammonia (Chen et al. 2008). Thus, pH and ammonium were measured at the end of the BMP test (Table 2) and free ammonia was calculated on the base of temperature, pH and ammonium concentration (Østergaard 1985). Free ammonia can be toxic at low concentrations (Li et al. 2016) because it easily goes through the bacteria's membrane (Chen et al. 2014).

At the end of the study the pH ranged between 7.50 and 7.78 in each studied mixture. The ammonium concentrations ranged between 1224±24 mg/L at the end of the BMP test of 100% *S. quadricauda* and 882±12 mg/L measured in 100% OMSW. The ammonium measured at the end of the BMP test of 100 % microalgae was lower than the limit established (1700-1800 mg/L) in the literature as toxic (Yenigün and Demirel 2013). As a consequence of pH range, it was expected that the predominant form of inorganic nitrogen was ammonium. Even so, free ammonia was calculated and it was demonstrated that the free ammonia was not a cause of methanogenesis inhibition. Ramos-Suárez et al.

(2014) observed that the degree of degradation of *Scenedesmus* cells was very low during the anaerobic co-digestion of *Opuntia maxima* and *Scenedesmus* cells. They found low fraction of N-NH_4^+ during anaerobic co-digestion. Mussgnug et al. (2010) showed that *Scenedesmus* sp. has hardly any biodegradable cell walls that prevent microorganisms from degrading their cell content. Solé-Bundó et al. (2018) did not find much more methane production in their trials despite their pre-treated microalgae to break their cell wall. In contrast, methane production increased when the hydraulic retention time was increased.

3.3 Methane yields and study of possible synergic effects

Figure 1 illustrates the variation in the specific cumulative methane production (mL CH_4 STP/g VS) with digestion time for the BMP assays carried out with 100% OMSW, 100% *S. quadricauda* and for the co-digestion mixtures 75% OMSW-25% *S. quadricauda*, 50% OMSW-50% *S. quadricauda* and 25% OMSW-75% *S. quadricauda*.

The highest methane yield after 20 days was 461 mL CH_4/g VS added for the co-digestion of 75% OMSW-25% *S. quadricauda*, while the methane yields obtained for the digestion of the sole substrates were 375 for the AD of the OMSW and 226 mL CH_4 STP/g VS added for the AD of the microalga. The value obtained for the BMP test of *S. quadricauda* was in accordance with previous studies (Mussgnug et al. 2010) Mussgnug et al. (2010)

reported *Scenedesmus* as a microalga with a low degree of decomposition and a high amount of indigestible residues. Nonetheless, the co-digestion of OMSW and *S. quadricauda* had a high efficiency in methane yield enhancement. The methane yield recorded by Fernández-Rodríguez et al. (2014) from the best co-digestion mixture of 75% OMSW- 25% *Dunaliella salina* was 330 mL CH_4/g VS added, lower than the best methane yield obtained in the present study for the co-digestion 75% OMSW -25% *S. quadricauda* (461 mL CH_4 STP/g VS added). Previous research has demonstrated that methane yield from microalgal biomass is highly variable and dependent on the strain used as substrate for anaerobic digestion as well as the growth conditions applied to generate the biomass (Mussgnug et al., 2010). Moreover, the use of saline strains, such as *Dunaliella salina* has shown that methane production decreases concomitantly with increasing salinity, such as occurred when *Dunaliella salina* (25%) was co-digested with OMSW (75%) (Fernández-Rodríguez et al., 2014).

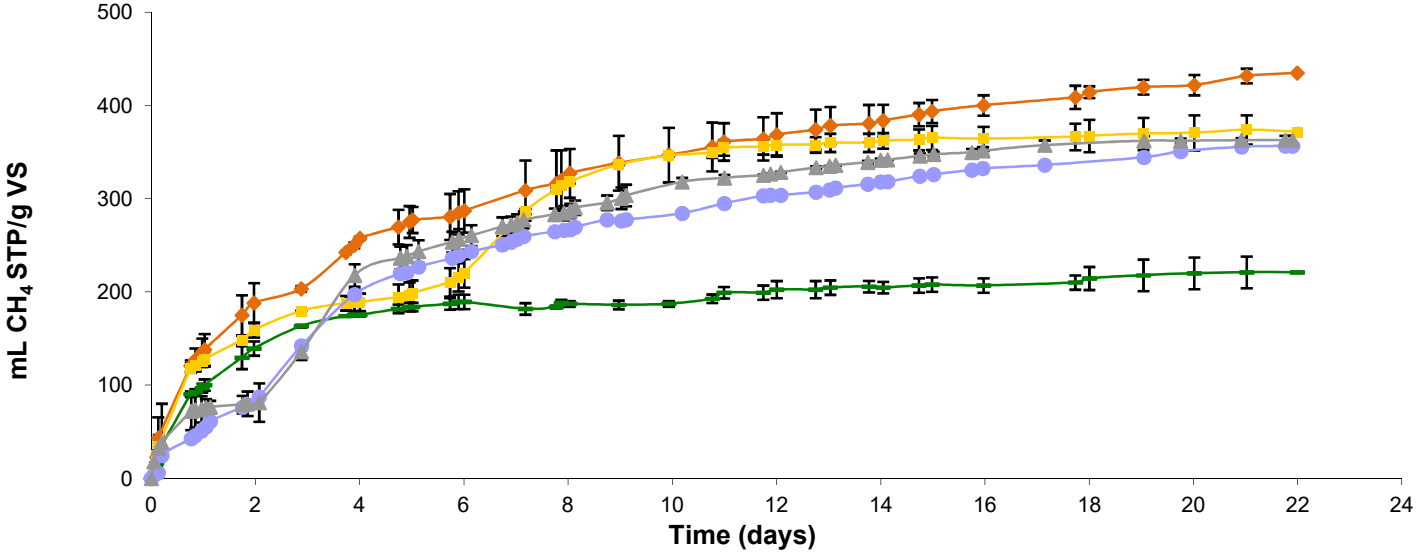

Figure 1. Biochemical methane potential (mL CH_4/g VS added) of 100% olive mill solid waste (OMSW) (■), 100% *Scenedesmus quadricauda* (-) and different co-digestion mixtures tested: 75% OMSW-25% *Scenedesmus quadricauda* (♦); 50% OMSW-50% *Scenedesmus quadricauda* (▲) and 25% OMSW- 75 % *Scenedesmus quadricauda* (●).

The experimental methane yields observed for each co-digestion mixture (Figure 1) were compared to calculated methane yields based on the OMSW, and *S. quadricauda* methane yields separately according to the equation (3).

$$\text{Calculated methane yield (mL } CH_4 \text{ STP/g } VS_{added}) = \text{\% OMSW*(375) + \% } \textit{S. quadricauda} \text{ *(226)} \quad (3)$$

Where 375 and 226 are the experimental methane yields (mL CH_4 STP/g VS_{added}) obtained for OMSW and *S. quadricauda*, respectively. % OMSW and % *S. quadricauda* are the percentages of OMSW and *S. quadricauda*, respectively, in each co-digestion mixture. Table 3 summarizes the experimental methane yields obtained for all experiments carried out, as well as the corresponding calculated ones.

Experimental BMP values were higher than the calculated methane yields from equation (3) for some of the co-digestion mixtures tested showing the occurrence of some synergistic effects (Table 3). For instance, 36.3% for the co-digestion mixture 75% OMSW-25% *S. quadricauda*, 20.6% for the co-digestion mixture 50% OMSW-50% *S. quadricauda* and 35.4 in the case of the mixture 25% OMSW-75% *S. quadricauda*. Therefore, according to the increase in the BMP values, the biodegradability of the above-

mentioned co-digestion mixtures was also much higher than the biodegradability of the sole substrates (Table 3) Ajeej et al. (2015) pointed out co-digestion as an effective system of enhancing substrate anaerobic biodegradability. An anaerobic co-digestion study showed that the addition of *Dunaliella salina* to OMSW improved the biodegradability of the sole substrate (Fernández-Rodríguez et al. 2014). In this study, only 58% of the available COD was converted to methane during the digestion of 100% OMSW. The co-digestion mixture 75% OMSW-25% *S. quadricauda* had a biodegradability of 89.5%, 91.8% and 93.2% for the co-digestion mixture 50% OMSW-50% *S. quadricauda* and 25% OMSW- 75% *S. quadricauda,* respectively. The biodegradability of the 100 *S. quadricauda* was only 80%. There were clear synergistic effects during the co-digestion of both substrates, since all co-digestions improved the biodegradability of the sole substrate. A clear decrease in the biodegradability was observed when the concentration of OMSW increased. However, despite the increase in microalgae, the biodegradability was similar in each co-digestion mixture.

Table 3. Calculated methane yield values obtained from equation (1), experimental data obtained through BMP test and biodegradability of the different co-digestion mixtures.

C/N ratio	**OMSW** (%)	***S.quadricauda*** (%)	**B_{max} Calculated** (mL CH_4 STP/g VS)	**B_{max} Experimental** (mL CH_4 STP/g VS)	**Methane yield improvement** (%)	**Biodegradability (COD-CH_4 / CODadded)** (%)
31.9	100	0	375	375	---	58.4
25.3	75	25	338	461	36.3	89.5
18.7	50	50	301	363	20.6	91.8
12.1	25	75	263	356	35.4	93.2
5.6	0	100	226	226	---	80

*OMSW: olive mill solid waste, *S.quadricauda: Scenedesmus quadricauda, B_{max}:* ultimate methane production

As can be seen in Table 3, the co-digestion mixture 75% OMSW-25% *S. quadricauda* was the combination of the highest methane yield, i.e. 461 mL CH_4 STP/g VS. This co-digestion mixture corresponded to a C/N ratio of 25.3, an intermediate value between 20/1 and 30/1 considered as the optimum C/N range in the literature (Habiba et al. 2009). In addition, the methane yield obtained from the mixture 75% OMSW-25% *S. quadricauda* was 27% and 29% higher than those achieved for the mixtures 50% OMSW-50% *S. quadricauda* and 25% OMSW-75% *S. quadricauda*, respectively. Furthermore, the above-mentioned methane yield obtained from the co-digestion mixture 75% OMSW-25% *S. quadricauda* was 23 and 104% higher than the mono-digestion of OMSW (C/N ratio of 31.9) and *S. quadricauda* (C/N ratio of 5.6), respectively.

It has been previously reported in the literature that the C/N ratio is an important factor for anaerobic digestion processes. For instance, Yen and Brune (2007) reported that the co-digestion of a mixture of algal biomass and waste paper (50% based on VS) to keep the C/N ratio in a range between 20-30 doubled the methane production rate with respect to the value achieved for the single algal substrate. In the same way, the anaerobic co-digestion of potato processing waste (PPW) and the microalgae *Chlorella vulgaris* showed a maximum methane yield of 226 mL CH_4/g VS, for which the

C/N ratio was 22.7 (Zhang et al. 2019), very similar to the optimum C/N ratio obtained in the present work (25.3) for the same co-digestion mixture (75% OMSW-25% *S. quadricauda*). It has also been reported that the maximum methane yield during the co-digestion of wheat straw (80%) and microalgal biomass (20%) grown in wastewater was found to be 289 mL CH_4/g VS for a C/N ratio of 26.4 (Solé-Bundó et al. 2017). These results also showed that the methane yield was increased by 77% with co-digestion as compared to microalgae mono-digestion. Other studies have demonstrated that co-digestion can also increase the anaerobic digestibility of microalgae by improving the substrate composition. Some co-substrates can have a co-effect in the sense that they stimulate enzymatic synthesis that can also improve the anaerobic digestion yield (Ajeej et al. 2015). In this sense, sewage sludge improves the digestibility of microalgae and enhances the production of methane when the C/N ratios of the co-digestion mixtures achieve values between 20-25 (Ajeej et al. 2015). Other studies have shown that the addition of septic sludge to the microalgae *Chlorella* sp. resulted in more favorable initial carbon to nitrogen ratios (C/N) (27:1), improved digestibility of algal biomass, and decreased hydrogen concentrations, which were directly related to the increased quantity and quality of the methane produced. These results demonstrated the effectiveness of using septic sludge as a co-substrate to anaerobically digest the

microalgae *Chlorella* sp. and enhance biogas production (Lu and Zhang 2016).

3.4 Kinetic study

Table 4 summarizes the parameters obtained from the application of the Transference Function to the experimental data shown in Figure 1.

Among the different co-digestion mixtures assayed, the highest maximum methane production rate, R_m, was obtained from the mixture 75% OMSW-25% *S. quadricauda* with a value of 89 mL CH_4/(g VS d). This value was 25% and 39% higher than those obtained for 50% OMSW-50% *S. quadricauda* and 25% OMSW-75% *S. quadricauda*, respectively. In addition, it was 20% higher than that achieved through single OMSW (100% OMSW).

More specifically, a decrease in the maximum methane production rate, R_m, from 89 to 64 mL CH_4/(g VS d) was observed when the percentage of *S. quadricauda* increased from 25% to 75%. In the same way, It was recently reported that the values for R_m obtained by the co-digestion of *Chlorella vulgaris* with potato processing waste (PPW) were gradually decreased as the proportions of *Chlorella* augmented in the mixture from 25% to 75% (on VS basis) (Zhang et al. 2019).

On the other hand, the lag periods found in all co-digestion mixtures tested in the present work were very low, and the shape of the curves of methane production with time was almost exponential

for all substrates tested. The ammonia release during theco-digestion of increased concentrations of *S. quadricauda* could explain the poorer digestion performance and slower kinetics when the percentages of this microalga in the co-digestion mixtures increased (Fernández-Rodríguez et al. 2014).

The co-digestion of microalgal biomass with primary sludge substantially improved the anaerobic digestion kinetics (k=0.25-0.28 days^{-1}) as compared to mono-digestion trials (k=0.07 days^{-1} for microalgae) (Solé-Bundó et al. 2018). Slight improvements in the degradation kinetics of the mixture of microalgae/bacteria biomass (MAB) grown on piggery wastewater (20%) with carbonaceous substrates (deproteinated cheese whey and cellulose) (20%) were observed compared to the digestion of the sole MAB (Carminati et al. 2018). In the same way, it was also reported that the anaerobic co-digestion of mixed microalgae (*Scenedesmus* sp. *and Chlorella* sp.) (MA) and food waste (FW) at a ratio of 20:80 increased the maximum methane production rate by 2.66 with respect to the microalgae alone (Zhen et al. 2016). The lag phase disappeared in the co-digestion trials; while it was 0.2 days for the pure microalgae. These results again reflect that the co-digestion of microalgae with carbon-rich co-substrates (i.e. food wastes) had a relatively high impact on microalgal anaerobic biodegradability and conversion rate (Zhen et al. 2016).

An implication of this study is that, anaerobic co-digestion of microalgae with OMSW is a promising technology for sustainable

energy production. Microalgae-bacterial consortium grown in wastewater could reduce costs and be a more sustainable alternative. Moreover, more information about the benefits of several co-substrates in the activity and performance of microbial population in anaerobic co-digestion is necessary. Deeply research in continuous operation is necessary focusing on novel reactor configuration designs that ensure low hydraulic retention times and high organic loading rates.

Table 4. Values for the parameters obtained from the **Transference Function** model for the different substrates studied.

Substrate	B_m (mL CH_4 STP/ g VS)	R_m (mL CH_4/ g VS d)	ʎ (d)	R^2	S.E.E.	Error** (%)
100% OMSW	381 ± 8	74 ± 5	0.0004	0.9705	27.48	1.6
75%OMSW-25% *S.q.*	421 ± 8	89 ± 5	0.0006	0.9763	26.72	8.6
50%OMSW-50% *S.q.*	367 ± 4	71 ± 2	0.0001	0.9944	12.36	1.1
25%OMSW-75% *S.q.*	351 ± 3	64 ± 2	0.045	0.9967	9.21	1.4
100% *S.q.*	205 ± 2	115 ± 8	0.0001	0.9869	12.02	9.2

*_S.q._: _Senedesmus quadricauda,_ OMSW_:_ Olive mil solid waste, _S.E.E._: Standard error of estimate, B_m: ultimate methane production, R_m: maximum methane production rate, S.E.E.: standard error of estimate, ʎ: calculated lag times

**Error $((B_{m\ experimental} - B_{m\ model})/B_{m\ experimental})\cdot 100$

4. Conclusions

These results confirmed the powerfulness of the co-digestion of carbon-rich OMSW with nitrogen-rich microalgae. Co-digestion increased the biodegradability of both substrates and the conversion rate. When the microalga *Scenedesmus quadricauda* was added to OMSW at a percentage of 75% OMSW- 25% *S. quadricauda* (C/N ratio=25.3), the methane yield and the methane production rate were improved compared to the anaerobic digestion of the sole substrates and other co-digestion mixture percentages. Co-digestion also helped the stability of the anaerobic digestion system. The microalga supplied nitrogen to the system, thus balancing the C/N ratio and providing extra alkalinity. The transference function model allowed for adequately fitting the experimental results of methane production with time in the anaerobic experiments. Among the different co-digestion mixtures assayed, the highest maximum methane production rate, R_m, was obtained from the mixture 75% OMSW-25% *S. quadricauda* with a value of 89 mL CH_4/(g VS d). This value was 25 and 39% higher than that obtained for 50% OMSW-50% *S. quadricauda* and 25% OMSW-75% *S. quadricauda*, respectively. In addition, this production rate was 20% higher than that achieved for single OMSW (100% OMSW).

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Chapter 5

Influence of the cell wall of *Chlamydomonas reinhardtii* on anaerobic digestion yield and on its anaerobic co-digestion with a carbon-rich substrate

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Abstract

The aim of this study was to investigate the influence of the cell wall of the microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*) on its anaerobic digestion (AD) by comparing the AD of *C. reinhardtii* 6145 and of its mutant without cell wall *C. reinhardtii* cw15, assessing simultaneously the influence of the cell wall on anaerobic co-digestion of these strains with a carbon-rich substrate, olive mill solid waste (OMSW). The OMSW is the main by-product from two-phase olive oil manufacturing process. Biochemical methane potential (BMP) tests of the sole substrates and different mixtures (OMSW-microalgae) (85%-15%, 75%-25%, 50%-50%) were carried out. The results showed that the cell wall was not hindrance and became an advantage by releasing intracellular biomass to be degraded slowly. The influence of the substrate composition in the mixtures of OMSW and *Chlamydomonas reinhardtii* (6145 and cw15) was assessed through the calculation of performance and kinetic parameters by using the Transference Function model. The mixture 50% OMSW-50% *C. reinhardtii* 6145 allowed the highest methane yield (542 ± 4 mL CH_4/g VS) and also resulted in one of the highest maximum methane production rates, R_{max} (129 ± 3 mL CH_4/(g VS·d).

1. Introduction

Green microalgae are considered to be a promising source of sustainable energy and/or high-value added products such as carotene, vitamins or fatty acids (Baudelet et al., 2017). Chlorophyta has been the focus of taxonomists, whose studies have led to a better understanding of its cell wall. Chlorophyta is the most prominent phylum with great interest in biotechnology and pharmaceuticals industries (Baudelet et al., 2017). The polyphyletic genus *Chlamydomonas*, within Chlorophyta family comprises biflagellate unicellular green alga which swims along a helical trajectory by the synchronous beating of its flagellar pair (Baudelet et al., 2017). The cell wall of *Chlamydomonas* consisting in a five-layered cell wall with no cellulose but is mainly composed of proteins linked by covalent bonds (Voigt and Frank, 2003).

The anaerobic digestion (AD) of microalgae in order to produce biogas is currently an alternative with low operational costs compared to other processes, with a wide range of biogas yields being reported, due to different factors: microalgae composition, cell wall structures or operational cultivation (Frigon et al., 2013). Among the bibliography is the object of consensus that microalgae as substrates for AD are directly related to cell wall digestibility, which depends on the content of the cellulose, hemicellulose and other hardly biodegradable biopolymers (Jankowska et al., 2017). Microalgae cell wall presents a high resistance to enzymatic hydrolysis and limits the availability for AD (Jankowska et al.,

2017). For this reason, several physical, chemical or biological pre-treatments have been evaluated in order to increase the biodegradability of the cell wall and, hence, enhance the methane production after AD (Jankowska et al., 2017). Mussgnug et al. (2010) showed that the hydrogen production of *C. reinhardtii* as a pre-treatment increased its biogas production potential by up to 123% (587 mL biogas/g VS). Passos et al. (2013) showed that a thermal pre-treatment increased the methane yield by up to 7 - 61% (170 - 272 mL CH_4/g VS). These results suggest that even in the case of a weak cell wall (like in *C. reinhardtii*) a pre-treatment is needed in order to enhance the AD process. However, Mahdy et al. (2014) achieved a methane yield of 263.1 mL CH_4/g COD in the AD of *C. reinhardtii* and this study concluded that the use of carbohydrases as a pre-treatment, which increased the solubilization of the organic matter, did not significantly increase the methane potential after AD. This could be explained by the lack of cellulose in the cell wall and because the released organic matter came from already easily bioavailable organic matter in the raw material. By contrast to this, when using proteases, the methane yield increased slightly (up to 311.6 mL CH_4/g COD). This can also be explained based on cell wall composition, which is mainly composed of glycoproteins (Mahdy et al., 2014).

Besides the cell wall, a major issue concerning the anaerobic digestion of any biomass is the C/N ratio of the substrate. A low C/N ratio, with containing a high level of nitrogen, produces an

intolerant concentration of ammonia for the correct activity of the microbial community within the anaerobic sludge, while a high C/N ratio, containing a low level of nitrogen, is related to a low pH substrate, poor buffering capacity and the volatile fatty acid accumulation (González-Fernández et al., 2011). Several authors have observed that the optimum C/N ratio of the substrate for AD with regards to higher pollutant and CO_2 removal must be between 20:1 and 30:1 (Fernández-Rodríguez et al., 2014; Xu et al., 2017). Since microalgal biomass presents a high nitrogen content, with C/N ratios ranging from 5 to 12 (Fernández-Rodríguez et al., 2014; Klassen et al., 2015), an additional source of carbon must be added in order to reach the optimal ratio. To this respect, co-digestion with high carbon biomass, like olive mill solid waste (OMSW), presents several improvements such as enhancing the C/N ratio, balancing the macro and micronutrient compositions, neutralizing the pH and reducing the effect of inhibitors/toxic compounds (Hartmann and Ahring, 2005; Li et al., 2018; Ferreira et al., 2018).

The OMSW is the main waste generated in the two-phase olive oil extraction process. This by-product is produced in an amount of 800 kg/t of olives processed. Its characteristics (high moisture, low pH, high content in organic matter, presence of inhibitory compounds such as poly-phenols, etc.) make it a very pollutant waste (de la Lama et al., 2017).

The potential of using microalgae as co-substrate of carbon-rich by-products has recently been reported in several studies.

González-Fernández et al. (2011) reported the lowest methane yield values when the microalgae percentage was increased in relation to a carbon-rich substrate, due to the highly resistant cell wall, stating that a pre-treatment method is required to improve microalgae digestibility. Another approach for anaerobic co-digestion of lignocellulosic residues with microalgae showed a synergistic effect during anaerobic co-digestion of both substrates, achieving the best methane production when the microalgae biomass was decreased and the lignocellulosic waste increased (Fernández-Rodríguez et al., 2014). Similar results were reported by Zhang et al. (2019) that obtained an increase in methane production of up to 47% with the lowest percentage of microalgae biomass during its co-digestion with potato processing waste.

The aim of this study was to determine the influence of non-rigid cell wall microalgae (*C. reinhardtii*) on an anaerobic digestion and co-digestion of these microalgae with a high carbon lignocellulosic biomass (OMSW). To this aim, two strains of *C. reinhardtii* were evaluated, 6145 and cw15. *C. reinhardtii* cw15 is a mutant specimen developed by Hyams and Davies (Hyams and Davies, 1972), by using N-methyl-N´-nitrosoguanidine (NNG) as mutagen which produces a phenotypic change where the cell wall is almost completely reduced. To the best of our knowledge, the influence of the cell wall of microalgae of the same species on their anaerobic digestion and anaerobic co-digestion of these with a

carbon-rich waste such as OMSW have not been previously reported.

2. Materials and methods

2.1 Olive mill solid waste

The two-phase OMSW was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). The OMSW was sifted through a 2 mm mesh with the purpose of removing olive stone pieces. The main characteristics of the OMSW used in the experiments were: pH: 4.9, total solids (TS): 256.7 ± 4.4 g/kg, volatile solids (VS): 226.2 ± 4.6 g/kg, chemical oxygen demand (COD): 345.3 ± 6.3 g O_2/kg and C/N ratio: 31.4 ± 0.1.

2.2 Microalgae cultivation

The chlorophytes *Chlamydomonas reinhardtii* 6145 and cw15 were grown at 25 ºC in a liquid medium. The microalgae were grown in the medium as described by Harris (1989). The algal biomass was cultivated under 12:8 light:dark cycles in an AGP-700-ESP incubator chamber (Rdiber S.A., Barcelona, Spain) with illumination provided by 6 fluorescent lamps (36 W). To ensure that the algal culture was composed mainly of *Chlamydomonas reinhardtii,* microscope observations were carried out periodically. The cells from the culture were concentrated by centrifugation (5 min at 3500 rpm). Finally, microalgal biomass was placed in liquid

nitrogen (-196 °*C*) and stored at -80 ºC. Table 1 shows the composition of both strains.

2.3 Microalgae chemical composition

The following parameters were determined to characterize the microalgal chemical composition: total solids and volatile solids were determined according to Standard Methods 2540B and 2540E (APHA, 1998), respectively; total lipids were determined by the Bligh and Dyer method (Bligh and Dyer, 1959); carbohydrates were quantified by Dubois method (Dubois et al., 1956). The concentrations of chlorophylls and carotenoids were measured at 663.2, 646.8, and 470 nm according to the standard method 10200H (APHA, 1998), and pigment concentrations were determined using the method described in detail elsewhere (Lichtenthaler, 1987). Fatty acids were extracted with Soxhlet equipment, with a mixture of chloroform and methanol (2:1) and were quantified spectrophotometrically at 628 nm by the reaction with sulfophosphovanillin (Ahlgren et al., 1992). Carbon and nitrogen contents were determined through an Elemental Analyzer LECO CHNS-932 (Leco Corporation, St Joseph, MI, E.E.U.U.).

2.4 Inocula for anaerobic digestion

The anaerobic sludge was obtained from an industrial up-flow anaerobic sludge blanket (UASB) reactor treating brewery wastewater in Sevilla (Spain). The main characteristics of the

inoculum used were: pH: 7.43; total solids (TS): 23.9 ± 1.1 g/L; volatile solids (VS): 18.7 ± 1.4 g/L; chemical oxygen demand (COD): 31.9 ± 1.1 g O_2/L.

2.5 Biochemical methane potential (BMP) tests

The tests were carried out in a multi-batch reactor system with an effective volume of 200 mL. The reactors were continuously agitated by magnetic bars at 440 rpm and placed in a thermostatic water bath at mesophilic temperature (35 ± 2 ºC).

The inoculum to substrate ratio was 2 (VS basis). Each reactor contained 192 mL of inoculum, the amount of substrate needed to give the required inoculum to substrate ratio and, 192 µL of trace element solution were added.

The composition of the trace elements solution was: $FeCl_2 \cdot 4H_2O$, 2000 mg/L; $CoCl_2 \cdot 6H_2O$, 2000 mg/L; $MnCl_2 \cdot 4H_2O$, 500 mg/L; $AlCl_3 \cdot 6H_2O$, 90 mg/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 mg/L; H_3BO_3, 50 mg/L; $ZnCl_2$, 50 mg/L; $CuCl_2 \cdot 2H_2O$, 38 mg/L, $NiCl_2 \cdot 6H_2O$, 50 mg/L, $Na_2SeO_3 \cdot 5H_2O$, 194 mg/L and EDTA 1000 mg/L. Two reactors with inoculum and trace element solution but without substrate addition were used as blank controls. The methane production due to biomass decay and the possible presence of residual substrate in the inoculum was subtracted by performing these blank controls. Therefore, the methane produced by the blank controls was subtracted from the reactors with substrate.

The reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 3N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. Thus, methane production was determined by liquid displacement. The anaerobic digestion experiments were run for a period of c.a. 29 days until the accumulated gas production remained essentially unchanged, i.e. on the last day production was lower than 2% of the accumulated methane produced. Each experiment was carried out in triplicate.

OMSW was co-digested with different percentages of both microalgal strains in order to compare the effect of cell wall on anaerobic co-digestion with OMSW. The co-digestion mixtures studied were the same for both microalgae, i.e. 85% OMSW-15% *C. reinhardtii*; 75% OMSW-25% *C. reinhardtii*; 50% OMSW-50% *C. reinhardtii*. The sole substrate OMSW and the single anaerobic digestion of *C. reinhardtii* 6145 and *C. reinhardtii* cw15 were also tested in order to show the effect of the cell wall on anaerobic digestion.

2.6 Analytical methods

All analyses were performed according to the Standard Methods of APHA (APHA, 1998). The following parameters were measured: total chemical oxygen demand (COD), soluble chemical oxygen demand (sCOD), total solids (TS), volatile solids (VS), pH, ammonium concentration ($N\text{-}NH_4^+$) and elemental C and N.

Soluble parameters were determined after sample centrifugation (eppendorf, 10000 rpm, 10 min) and filtration (glass fiber filter 47 mm).

TS and VS were determined according to the standard methods 2540B and 2540E (APHA, 1998), respectively; COD was determined by the method described in detail elsewhere (Raposo et al., 2008); while sCOD was determined using the closed digestion and the colorimetric standard method 5220D (APHA, 1998). pH was analyzed using a pH-meter model Crison 20 Basic. Total alkalinity (TA) was determined by pH titration to 4.3 (APHA, 1998). Total ammonium was determined using a method based on 4500-N_{org} B of Standard Methods (APHA, 1998). Free ammonia concentration was calculated according to the following formula (Østergaard, 1985):

$$\frac{[NH_3]}{[TNH_3]} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}}\right)^{-1} \quad (1)$$

where $[NH_3]$ is the concentration of free ammonia, $[TNH_3]$ is the total ammonia concentration and T(K) is the temperature measured in degrees kelvin.

Individual volatile fatty acids (VFA) from C2 to C7 including iso-C4, iso-C5 and iso-C6 were analyzed using a Gas

Chromatograph (Shimadzu GC 2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol (polyethylene glycol modified by nitroterephthalic acid). Prior to injection, 900 µL of the sample were mixed with 150 µL of H_3PO_4 (1:2 v/v) to adjust the pH to below 2.0 and 150 µL of a solution of crotonic acid (2000 mg/L) as an internal standard. This mixture was centrifuged to remove any solids and transferred to a 1500 µL gas chromatography (GC) vial; the sample injection volume was 1 µL. The temperatures of the injector and detector were maintained at 200 ºC and 250 ºC, respectively, while the column temperature was increased from 120 to 160 ºC at an increasing rate of 10 ºC/min. Total phenols concentration was quantified by spectrophotometry through the Folin-Ciocalteau method (de la Lama et al., 2017).

2.7 Statistical analysis

Data are presented as means ± standard deviation of the means, and statistical significances were assessed by analysis of variance (ANOVA). A p value of less than 0.05 was considered statistically significant.

2.8 Kinetic study

The Transference Function (TF) model was applied to fit the experimental data of methane production during BMP tests (eq. 2). The transference function (Reaction curve-type model) (RC), used

mainly for control purposes, considers that any process might be analyzed as a system receiving inputs and generating outputs (Donoso-Bravo et al., 2010). The TF model was successfully applied by several authors for biomethanization of different organic wastes (Donoso-Bravo et al., 2010; Li et al., 2012). The TF model is given by the following expression:

$$B = B_{max} * \left(1 - exp\left[-\frac{R_{max}(t-\gamma)}{B_{max}}\right]\right) \quad (2)$$

where B (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, R_{max} is the maximum methane production rate (mL CH_4/(g VS_{added}·d)), t (d) is the digestion time and γ (d) is the lag time.

Error (%), determination coefficient (R^2) and standard error of estimate were calculated to evaluate the goodness-of-fit and the accuracy of the results. Error was defined as the percentage difference between the experimental and the predicted or theoretical methane yield coefficient. The kinetic parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 11).

3. Results and discussion

3.1 Chemical composition of *C. reinhardtii*

Several studies have recommended the application of pre-treatments on microalgae in order to enhance their biodegradability and, therefore, their bioconversion into methane (Passos et al., 2014). However, it is known that methane production strongly depends on the microalga type (Mussgnug et al., 2010). As discussed previously, *C. reinhardtii* does not present cellulose in its cell wall, which consists instead of a five-layered extracellular matrix composed of carbohydrates and polypeptides (Voigt and Frank, 2003).

In order to test the effect of this type of cell wall in AD processes, two different types of *C. reinhardtii* were used, i.e. a wild type (6145) and a mutant type (cw15). cw15 strain type is a mutant that differs from the 6145 type in the absence of cell wall by using N-methyl-N′-nitrosoguanidine (NNG) as mutagen which produces a phenotypic change (Hyams and Davies, 1972).

The chemical composition of the microalgae depends on growth conditions, harvesting time and conservation technology (Wang and Park, 2015). Consequently, as both strains have been cultivated under the same conditions, i.e. harvested at same time and conserved with the same technology, they presented similar chemical characteristics with no significant differences (p<0.005).

As shown in Table 1, the lipid contents were 20% and 21% on VS basis for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. The protein and carbohydrate contents were 50% and 16% on VS basis for both cases, respectively. The chlorophyl contents were 212 and 209 µg/L for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. And the C/N ratio for both strains was 5.2. Similar results were obtained by Mahdy et al. (2014), which showed a concentration of lipids, protein and carbohydrates of 126.0 mg/$g_{dry\ weigth}$, 647.6 ± 8.0 mg/$g_{dry\ weigth}$ and 226.4 ± 21.9 mg/$g_{dry\ weigth}$, respectively, for a *C. reinhardtii* wild type. The low C/N ratio of both strains, which implies a high N content, suggests that an anaerobic co-digestion with a high carbon co-substrate like OMSW will result in higher methane production.

Table 1. Chemical characteristics of the different microalgal strains *Chlamydomonas reinhardtii* 6145 (*Ch.r.* 6145) and *Chlamydomonas reinhardtii* cw15 (*Ch.r.* cw15) before anaerobic digestion.

Parameter	**Ch.r.6145**	**Ch.r.cw15**
Total Solid (g/L)	57.6 ± 5.4	57.4 ± 2.2
Volatile Solids (g/L)	57.1 ± 2.4	56.2 ± 3.3
Chemical Oxygen Demand (g O_2/L)	49.4 ± 0.0	58.1 ± 8.7
H (%)	6.73 ± 0.23	6.86 ± 0.42
C/N	5.2 ± 0.0	5.2 ± 0.1
Proteins (%)	50±1	49±1
Lipids (%)	20±1	21±1
Carbohydrates (%)	16±1	16±1
Chlorophylls (µg/L)	212±20	209±12
Carotenoids (µg/L)	154±14	155±17

3.2 Nitrogen and volatile fatty acids inhibition

Due to the high nitrogen content of microalgae (5.2 C/N ratio) it was expected that methane production would be inhibited by one of its main methanogen inhibitors, the free ammonia, which can be toxic at concentrations as low as 2000 mg/L (Yenigün and Demirel, 2013). Although Wang et al. (2012) pointed out the 4.4 C/N ratio as the limit ratio to have ammonium inhibition, pH and ammonium were measured at the end of each BMP test and free ammonia was calculated on the base of temperature, pH and total ammonium concentration, in order to confirm that any inhibitory effect appeared was not related to ammonia toxicity (Table 2).

At the end of the study, the pH ranged between 7.66 and 8.12 and the total ammonium concentration ranged between 1.67 ± 0.07 g/L (100% *C. reinhardtii* cw15) and 0.94 ± 0.02 g/L (85% OMSW-15% *C. reinhardtii* 6145). These results showed that in no case the minimum concentrations for inhibitory effects were reached and, due to the pH range, it was expected that the predominant form of inorganic nitrogen was ammonium (Hansen et al., 1998). Even so, free ammonia was also calculated by using eq.1. The results ranged between 187.0 ± 7.8 mg/L (100% *C. reinhardtii* cw15) and 81.9 ± 6.3 mg/L (75% OMSW-25% *C. reinhardtii* cw45). Hansen et al. (1998) concluded that the value found for the inhibition of the biogas process was 1100 mg/L free ammonia. The free ammonia values calculated in these experiments were drastically lower than

the inhibitory limit; hence, free ammonia was not the cause of methanogenesis inhibition in any case.

The free ammonia content at the end of the experiments of the different co-digested mixtures were lower than in the AD of 100% *C. reinhardtii* 6145 and *C. reinhardtii* cw15. These results are consistent with others previously reported, i.e. Jianlong and Jing (2005) showed a reduction of 40% total ammonia when the initial concentration was 70-250 mg/L using an expanded granular bed reactor initiated by feeding the anaerobic sludge from a brewery wastewater treatment plant.

When comparing both microalgal strains no significant differences were observed for total ammonia except in the mixture 50% OMSW-50% *C. reinhardtii*, where the experiment with the wild type 6145 presented a concentration 33% higher than the same mixture with the mutant type cw15. By contrast, the smallest difference observed for the free ammonia was found in the 50% OMSW-50% *C. reinhardtii* where the wild type 6145 mixture presented a content only 4% higher than the same mixture with the mutant type cw15. In the single microalga experiments and in the mixture 85% OMSW-15% *C. reinhardtii*, the mutant type cw15 presented concentrations which were 10 and 12% higher, respectively, than observed for the wild type 6145. The mixture 75% OMSW-25% *C. reinhardtii* showed the greatest difference between both microalgae, with 21% higher concentration found in the mixture with the wild type 6145.

Table 2. Chemical composition of the Biochemical Methane Potential (BMP) test effluents.

Substrate	pH	Volatile Solids (g/L)	Soluble Phenols (ppm)	Soluble Sugars (ppm)	sCOD (gO_2/L)	COD (gO_2/L)	Total N-NH_4^+ (g/L)	Free N-NH_4^+ (mg/L)
100% *Ch. r.* 6145	8.02 ± 0.04	17.63 ± 0.59	347 ± 51	17.42 ± 1.33	6.53 ± 0.57	23.38 ± 1.48	1.62 ± 0.08	170.59 ± 8.42
100% *Ch. r.* cw15	8.05 ± 0.02	17.58 ± 1.42	311 ± 54	18.44 ± 4.67	6.27 ± 0.80	25.82 ± 4.13	1.67 ± 0,07	187.02 ± 7.84
100% OMSW	7.91 ± 0.16	22.88 ± 2.43	311 ± 40	11.27 ± 4.67	4.32 ± 1.04	27.76 ± 1.57	1.14 ± 0.12	95.43 ± 10.05
85%OMSW-15% *Ch.r.* 6145	7.97 ± 0.06	17.84 ± 1.11	275 ± 20	8.94 ± 1.80	3.12 ± 0.42	24.66 ± 1.42	0.94 ± 0.02	89.24 ± 1.90
85%OMSW-15% *Ch.r.* cw15	8.00 ± 0.11	18.89 ± 0.77	258 ± 25	7.18 ± 1.50	3.12 ± 0.51	25.01 ± 2.27	0.99 ± 0.02	100.03 ± 2.02
75%OMSW-25% *Ch.r.* 6145	7.90 ± 0.04	21.37 ± 0.50	219 ± 19	7.82 ± 0.97	3.48 ± 0.84	26.78 ± 1.51	1.23 ± 0.11	100.81 ± 9.02
75%OMSW-25% *Ch.r.* cw15	7.83 ± 0.15	21.73 ± 1.45	232 ± 22	7.76 ± 1.46	3.01 ± 0.36	27.14 ± 4.17	1.16 ± 0.09	81.92 ± 6.36
50%OMSW-50% *Ch.r.* 6145	7.94 ± 0.11	22.24 ± 1.50	281 ± 33	14.83 ± 1.75	5.12 ± 0.87	28.51 ± 0.47	1.74 ± 0.03	155.15 ± 2.67
50%OMSW-50% *Ch.r.* cw15	8.06 ± 0.03	18.98 ± 0.65	250 ± 5	13.98 ± 1.86	5.06 ± 0.27	26.83 ± 4.94	1.30 ± 0.23	148.59 ± 26.29

**Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15, OMSW: olive mill solid waste, sCOD: soluble chemical oxygen demand, COD: chemical oxygen demand

VFA were also tested in order to evaluate whether there was any inhibitory accumulation due to the unbalance of acidogenic and methanogenic reactions. VFA accumulation is the main consequence of process overloading which can acidify the reactor and hence inhibit the process. Table 3 showed the individual fatty acids determined. The single OMSW and the co-digested mixtures only presented acetic acid, the smaller VFA, which means that the acidogenic step was not inhibited. Moreover, the acetic acid concentrations were lower than 425 ppm for these cases, which suggest that no significant accumulation had taken place and that the methanogenic microorganisms were not totally inhibited by VFA. Franke-Whittle et al. (2014) reported that acetic acid and butyric acid concentrations of 2400 and 1800 mg/L respectively, resulted in no significant inhibition of the activity of methanogens, while a propionic acid concentration of 900 mg/L resulted in significant inhibition of the methanogens. By contrast, a much higher concentration of acetic acid was found in the single digestion of *C. reinhardtii* (1221.1 ± 16.0 ppm and 1074.3 ± 1.8 ppm for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively). Moreover, in these single microalga digestions, propionic acid was found.

Table 3. Volatile fatty acids of the different effluents measured by gas chromatography.

Substrate	Acetic Acid (ppm)	Propionic Acid (ppm)
100% *Ch.r.*6145	1221.1 ± 16.0	30.5 ± 1.6
100% *Ch.r.*cw15	1074.3 ± 1.8	14.6 ± 1.7
100% OMSW	81.0 ± 11.6	NF
85% OMSW-15% *Ch.r.*6145	58.9 ± 0.7	NF
85% OMSW-15% *Ch.r.*cw15	44.3 ± 3.0	NF
75% OMSW-25% *Ch.r.*6145	44.8 ± 2.8	NF
75% OMSW-25% *Ch.r.*cw15	42.1 ± 1.5	NF
50% OMSW-50% *Ch.r.*6145	102.4 ± 1.1	NF
50% OMSW-50% *Ch.r.*cw15	425.0 ± 30.0	NF

*NF: not founded, *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15 and OMSW: olive mill solid waste

These results suggest that the acidogenic and the methanogenic steps showed certain inhibition, due to the accumulation of VFA while the methane productions decayed to zero. Siegert and Banks (2005) reported similar results, concluding that biogas production using paper as substrate was reduced to half when the concentration of initial VFA was 1000 mg/L, indicating inhibition of the hydrolysis process.

3.3 Biodegradability

An important parameter to reflect anaerobic digestion efficiency is the biodegradability of the substrate. Many authors pointed out the low biodegradability of the cell wall of microalgae due to the rigid cell wall mainly composed of cellulose, hemicellulose and other hardly biodegradable biopolymers (Jankowska et al., 2017). In contrast, the insolubilization of the cell wall of *C. reinhardtii* is mainly due to covalent bonds between glycoproteins (isodityrosine and dityrosine, mainly), protease resistant isopeptide bonds and oligosaccharide side chain cross-link. Thus, as shown in Figure 1, the biodegradability (VS basis) of both strains was greater than 88%. These results were higher than those previously observed by Mahdy et al. (2014) who reported biodegradability of 75% using *C. reinhardtii* and similar to the 86% of the *C. vulgaris*.

Figure 1, also shows the biodegradability of the single digestion of OMSW and the different mixtures that were under study. As shown, OMSW presented the lowest biodegradability (56.7%) due

to the presence of non-easily-biodegradable compounds such as cellulose, hemicellulose and lignin which could represent up to the 30% of the dry weight (de la Lama et al., 2017).

The biodegradability of the mixture 75% OMSW-25% *C. reinhardtii* was similar for both species (65-67%). The mixtures 85% OMSW-15% *C. reinhardtii* presented similar values and the highest biodegradability (89.06% and 81.60% for the wild type 6145 and the mutant type cw15, respectively). By contrast, the mixtures 50% OMSW-50% C. reinhardtii showed greater differences between microalga strains, which were 81.28% for cw15 and 61.12% for 6145. Similar biodegradability values were found by Fernández-Rodríguez et al. (2014) who reported a maximum biodegradability of 73% when *Dunaliella salina* was co-digested with OMSW at a ratio of 1:1. Furthermore, as the OMSW presented lower biodegradability than the microalgae, it was expected that the higher the microalga percentage in the mixture the higher the biodegradability would be. However, results showed that the highest biodegradability was obtained with the lowest microalga percentage, which suggests a synergic effect between both substrates and a better C/N balance in the mixture 85% OMSW – 15% *C. reinhardtii.*

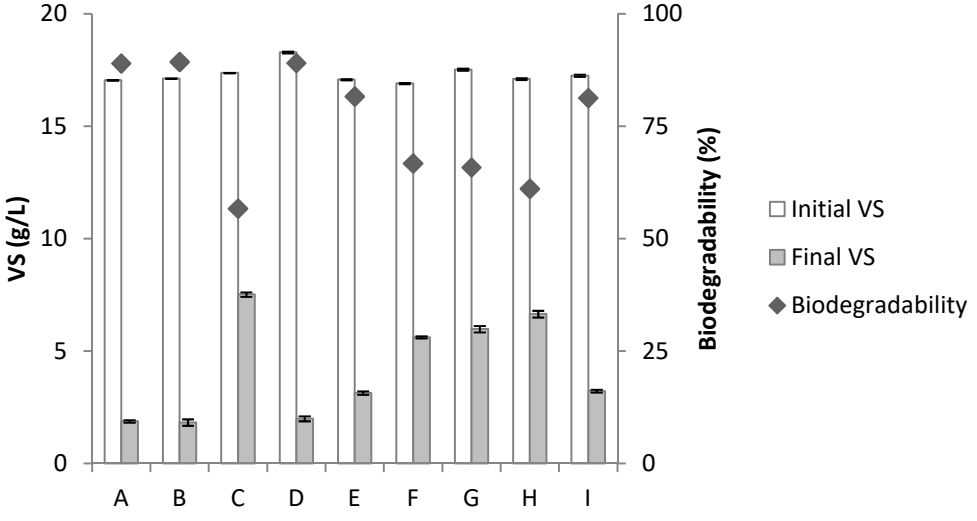

Figure 1. Biodegradability of the different tests carried out. Biodegradability was calculated by the difference between the initial Volatile Solids (VS) and the final VS on a percentage basis. A: *Chlamydomonas reinhardtii* 6145 (Ch.r.6145); B: *Chlamydomonas reinhardtii* cw15 (Ch.r.cw15); C: olive mill solid waste (OMSW); D: 85% OMSW-15% Ch.r.6145; E: 85% OMSW-15% Ch.r.cw15; F: 75% OMSW-25% Ch.r.6145; G: 75% OMSW-25% Ch.r.cw15; H: 50% OMSW-50% Ch.r.6145; I: 50% OMSW-50% Ch.r.cw15.

3.4 Effect of cell wall on anaerobic digestion

Figure 2a shows the mean values for the accumulated methane productions during the experimental time (29 days) for the two different microalgae tested, *C. reinhardtii* 6145 (with cell wall) and *C. reinhardtii* cw15 (the mutant without cell wall). As can be seen, the methane production increased after a short lag phase and an inflection point on the 5th day of digestion time for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively, up to a maximum value at the end of the digestion period. During the first four days of digestion both microalgae exhibited the same methane yield, but after 5 days the trend in the variation of methane production with time was somewhat different. At the end of the experiment, the maximum methane production reached by the microalga without cell wall was 381±37 mL CH_4/g VS, while the microalga *C. reinhardtii* 6145 with cell wall gave a final methane production of 351±39 mL CH_4/g VS. Therefore, the methane yield for the *C. reinhardtii* cw15 only increased by 8.5% in relation to the value obtained for *C. reinhardtii* 6145. Thus, the differences in these methane yields were not statistically significant. However, the shape of the curves of methane production with time revealed that the mutant without cell wall, *C. reinhardtii* cw15, was more bioavailable.

The experimental methane yield obtained for *C. reinhardtii* cw15 in the present work (381±37 mL CH_4/g VS) was virtually identical to the value reported by Mussgnug et al. (2010) for *C.*

reinhardtii (387 mL CH_4/g VS) and higher than that reported by Frigon et al. (2013) in this case using *Chlamydomonas* sp.-AMLS1b3 (333 mL CH_4/g VS) and *Chlamydomonas debaryana*-AMB1 (302 mL CH_4/g VS) as substrates for anaerobic digestion. The lower methane yields observed in the reported studies can most likely be attributed to the fact that different *Chlamydomonas* strains were used in the mentioned studies and/or that strict phototrophic cultivation conditions were applied in them. Therefore, this work clearly shows that methane yield from microalgal biomass is highly variable and dependent on the strain used as substrate for digestion as well as the growth conditions applied to generate the biomass (Mussgnug et al., 2010).

On the other hand, by comparing the two species of *C. reinhardtii* used in this work (*C. reinhardtii* 6145 and *C. reinhardtii* cw15), it can be seen that the species with a high degree of decomposition and low amount of indigestible residues or cell wall (*C. reinhardtii* cw15) showed somewhat higher methane yield (381 mL CH_4/g VS) compared to the species with a lower degree of decomposition and higher amount of indigestible residues (*C. reinhardtii* 6145) (351 mL CH_4/g VS). Consequently, these results indicate that without a pre-treatment, the accessibility to cell disintegration is most likely a major factor for the efficiency of fermentative methane production. As a consequence, the species with a very weak cell wall composed basically of protein with no cellulose or hemicellulose (*C. reinhardtii* cw15) generated a higher

methane yield compared to the species *C. reinhardtii* 6145 with a more rigid cell wall containing some biopolymers.

It has also been reported that the enzymatic hydrolysis of *C. reinhardtii* with carbohydrolase (Viscozyme) and protease (Alcalase) resulted in high carbohydrates and protein solubilization of this biomass. Despite the high carbohydrate solubilization, methane production was not improved after this pre-treatment, while the addition of protease increased methane production only by 1.17-fold (Mahdy et al., 2014). By contrast, a novel, one-stage combined cultivation/anaerobic fermentation strategy including inherently progressing nitrogen starvation conditions to generate improved microalgal biomass substrates allowed for increasing the C/N ratio of *C. reinhardtii* biomass up to levels of 24-26. This nitrogen limitation treatment resulted in a 65% increase in biogas yield for *C. reinhardtii* (698 mL biogas/g VS) when compared to biomass grown under replete conditions (Klassen et al., 2015). Finally, to evaluate integrative biorefinery concepts, biosolar hydrogen production in *C. reinhardtii* prior to anaerobic digestion resulted in an increase of biogas production to 123% compared to the fermentation of fresh untreated *C. reinhardtii* (Mussgnug et al., 2010).

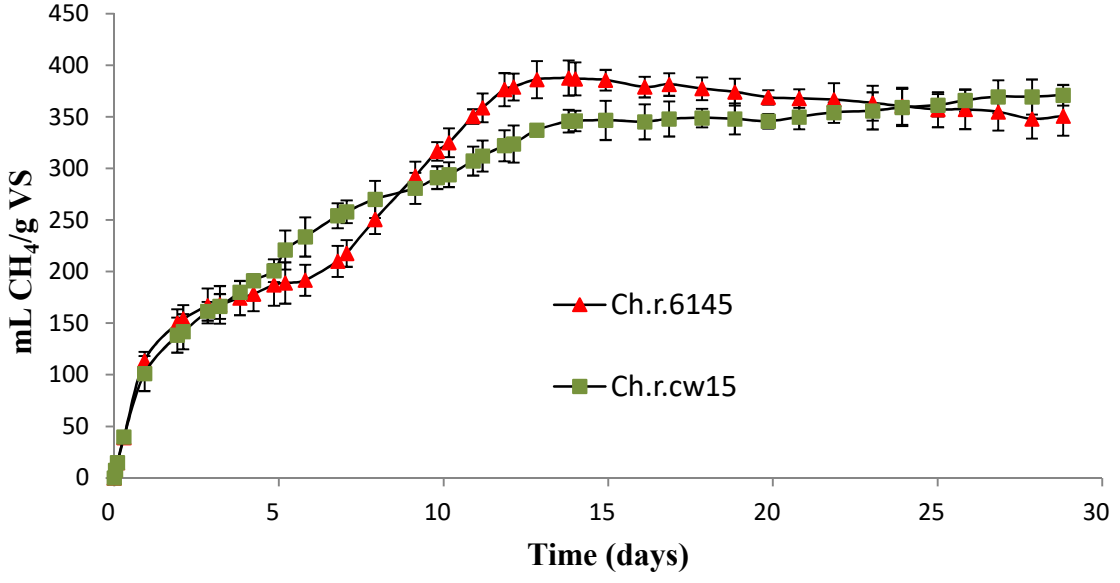

Figure 2a. Accumulated methane of single microalgae anaerobic digestion. *Chlamydomonas reinhardtii* 6145 (Ch.r. 6145) and *Chlamydomonas reinhardtii* cw15 (Ch.r. cw15).

3.5 Effect of different C. reinhardtii 6145-OMSW and C. reinhardtii cw15-OMSW mixtures on methane production

Figure 2b shows the variation in the mean values of the accumulated methane production with digestion time for the single OMSW substrate and different *C. reinhardtii* 6145-OMSW and *C. reinhardtii* cw15-OMSW mixtures tested.

For the co-digestion of OMSW with *C. reinhardtii* cw15 the maximum methane yield was achieved for the mixture 50% OMSW-50% *C. reinhardtii* cw15 (451 ± 36 mL CH_4/g VS added), which was 18% higher than that obtained for the single anaerobic digestion of 100% *C. reinhardtii* cw15 and 32% and 31% higher than that achieved for the mixtures 75% OMSW-25% *C. reinhardtii* cw15 and 85% OMSW-15% *C. reinhardtii* cw15 (341 ± 15 and 342 ± 47 mL CH_4/g VS), respectively. Different effects could be observed when comparing the results from the co-digestion 85% OMSW-15% *C. reinhardtii* cw15 and 75% OMSW-25% *C. reinhardtii* cw15 with this single microalga, since neither of these two mixtures improved the amount of methane obtained in relation to the anaerobic digestion of the sole microalga.

During the co-digestion of OMSW with *C. reinhardtii* 6145 (cell wall microalgae), the highest methane production was reached for the mixture 50% OMSW-50% *C. reinhardtii* 6145 (542 ± 38 mL CH_4/g VS), which was 54% higher than that achieved for the single digestion of *C. reinhardtii* 6145. Despite the C/N values considered optimal in the literature varies between 20 to 30, other

mixtures such as processed industrial food waste (IFW) with sewage sludge showed optimum methane yield at a C/N ratio of 15 (Benn and Zitomer, 2018), which is very similar to the C/N ratio of the mixture 50% OMSW – 50% *C. reinhardtii* 6145 (18.3) tested in the present work (Table 4). On the other hand, anaerobic co-digestion experiments of algal biomass and paper waste also demonstrated that the highest methane yield obtained in the above-mentioned mixture was attributed to the fact that microalgae were not only a nitrogen source supplier in this co-digestion, but also supplied nutrients to the digester microflora after the degradation of algal biomass (Yen and Brune, 2007).

The methane yield obtained for the digestion of the sole OMSW was 415 ± 34 mL CH_4/g VS added while the final methane yield for the single microalga (*C. reinhardtii* 6145) was 351 ± 39 CH_4/g VS added. The methane yield values obtained during the co-digestion of *C. reinhardtii* 6145 were 308 ± 3 mL CH_4/g VS added for the mixture 85% OMSW-15% *C. reinhardtii* 6145 and 421 ±16 mL CH_4/g VS added for the mixture 75% OMSW-25% *C. reinhardtii* 6145. Therefore, only the co-digestions of 50% OMSW-50% *C. reinhardtii* 6145 and 75% OMSW-25% *C. reinhardtii* 6145 showed enhancement in the methane yield of 54% and 20%, respectively, with respect to the value obtained for the single microalga (351 ± 39 mL CH_4/g VS added).

The results obtained suggest that the cell wall of this microalga had no negative effect during its anaerobic co-digestion with OMSW. In fact, the methane productions obtained during the co-digestion of the microalga with cell wall (*C. reinhardtii* 6145) with OMSW was significantly better than those obtained during the co-digestion of OMSW with the mutant microalga without cell wall (*C. reinhardtii* cw15).

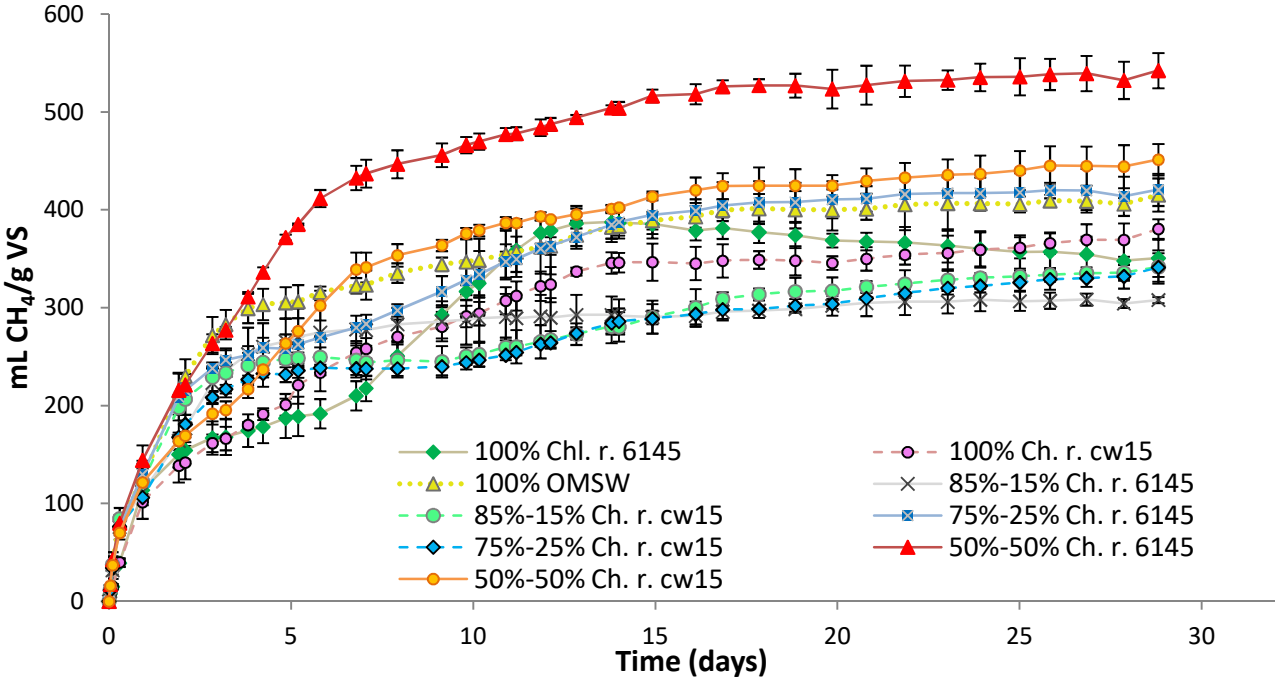

Figure 2b. Accumulated methane of single olive mill solid waste (OMSW) digestion and different co-digestion mixtures. Ch.r.: *Chlamydomonas reinhardtii*, OMSW: olive mil solid waste

3.6 Study of possible synergic effects

The experimental methane yields observed for each co-digestion mixture (Figure 2b) were compared to calculated methane yields based on the OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15 methane yields separately according to equations (3) and (4).

Calculated methane yield (mL CH_4/g VS_{added}) =
% OMSW*(415) + % *C. reinhardtii* 6145 *(351) (3)

Calculated methane yield (mL CH_4/g VS_{added}) =
% OMSW*(415) + % *C. reinhardtii* cw15 *(381) (4)

where 415, 351 and 381 are the experimental methane yields (mL CH_4/g VS_{added}) obtained for OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. % OMSW, % *C. reinhardtii* 6145 and % *C. reinhardtii* cw15 are the percentages of OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively, in each co-digestion mixture.

Table 4. Calculated methane yield values obtained from equations (3) and (4), experimental data obtained from Biochemical Methane Potential (BMP) tests of the different co-digestion mixtures.

Substrate	C/N ratio	B_{max} calculated (mL CH_4/g VS)	B_{max} experimental (mL CH_4/g VS)	Methane yield improvement (%)
85%OMSW-15% *Ch.r.* 6145	27.5	405	308	---
85%OMSW-15% *Ch.r.* cw15	27.5	410	342	---
75%OMSW-25% *Ch.r.* 6145	24.8	399	421	5.5%
75%OMSW-25% *Ch.r.* cw15	24.8	406	341	**---**
50%OMSW-50% *Ch.r.* 6145	18.3	383	542	41.5%
50%OMSW-50% *Ch.r.* cw15	18.3	398	451	13.3%

***** *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* OMSW: olive mill solid waste and B_{max}: ultimate methane production

Experimental BMP values were higher than the calculated methane yields for some of the co-digestion mixtures tested (Table 4). 5.5% for the co-digestion mixture 75% OMSW-25% *C. reinhardtii* 6145, 41.5% for the co-digestion mixture 50% OMSW-50% *C. reinhardtii* 6145 and 13.3% for the co-digestion mixture 50% OMSW-50% *C. reinhardtii* cw15. Therefore, according to the increase in the BMP values, the biodegradability of the above-mentioned co-digestion mixtures was also much higher than the biodegradability of the sole substrates. The synergy effects of the OMSW and *C. reinhardtii* 6145 and of OMSW and *C. reinhardtii* cw15 co-digestions with the above percentages were clearly shown with these results.

3.7 Kinetics of the single anaerobic digestion and co-digestion processes

Table 5 shows the main performance and kinetic parameters obtained from the application of the Transference model to the experimental data of methane production-time corresponding to the different BMPs of all the mixtures and single substrates carried out. As highlighted, the high R^2 values as well as the low values of errors and standard errors of estimates indicated that the experimental data correctly fit the proposed model.

The calculated lag times (ʎ) were found to be zero in all cases, because the easiest and most available biodegradable components

of all the substrates were quickly consumed in all the anaerobic digestion processes studied (Li et al., 2012).

The R_{max} values presented a somewhat different behavior and trend to that observed for B_{max} for the different experiments carried out. The highest R_{max} value was found for the mixture 85% OMSW-15% *C. reinhardtii* 6145 with 139 ± 5 mL CH_4/(g VS·d), mixture that also showed the highest biodegradability value (89%). This R_{max} value was 2.2% and 110.6% higher than that obtained for 100% OMSW (136 ± 8 mL CH_4/(g VS·d) and 100% *C. reinhardtii* 6145 (66 ± 5 mL CH_4/(g VS·d), respectively. High R_{max} values were also obtained for the mixtures 85% OMSW-15% *C. reinhardtii* cw15 (133 ± 16 mL CH_4/(g VS·d)) and 50% OMSW-50% *C. reinhardtii* 6145 (129 ± 3 mL CH_4/(g VS·d)). By contrast, the lowest R_{max} value was found for the mixture 50% OMSW-50% *C. reinhardtii* cw15 (88 ± 3 mL CH_4/(g VS·d)). This decrease in R_{max} for the mentioned mixture is a good indication that compounds in this mixture might have a lower initial availability for its anaerobic biodegradation. On the other hand, the R_{max} values obtained in the present work were always higher than those obtained during the co-digestion of OMSW and *Dunaliella salina*, for which the maximum value (48.1 mL CH_4/(g VS·d)) was obtained for the mixture 75% OMSW-25% *D. salina*. This fact reveals the higher anaerobic biodegradability of *C. reinhardtii* 6145 and *C. reinhardtii* cw15 compared to *D. salina* either alone or in mixtures with OMSW (Fernández-Rodríguez et al., 2014).

Finally, taking into account both the B_{max} and R_{max} values as well as the synergy effect observed, the mixture 50% OMSW-50% *C. reinhardtii* 6145 can be considered as the optimum one among the different mixtures tested in the present work.

4. Conclusions

It has been demonstrated that the cell wall of the microalga *C. reinhardtii* 6145 had no negative effect during its anaerobic co-digestion with OMSW. The methane production obtained during the co-digestion of this microalga with cell wall with OMSW was better than that obtained during the co-digestion of OMSW with the mutant microalga without cell wall (*C. reinhardtii* cw15). The mixture 50% OMSW-50% C. reinhardtii 6145, increased the methane yield compared to the anaerobic digestion of the sole substrates and other co-digestion mixtures percentages. Anaerobic co-digestion of 85% OMSW-15% C. reinhardtii 6145 resulted in the highest maximum methane production rate.

Table 5. Kinetic parameters obtained from the Transference function model applied to the different Biochemical Methane Potential (BMP) assays of single substrates and mixtures carried out.

Substrate	B_{max} (mL CH_4/g VS)	R_{max} (mL CH_4/g VS·d))	λ (d)	R^2	S.E.E.*	Error** (%)
100% *Ch.r.* 61445	383 ± 9	66 ± 5	0	0.9695	30.43	9.1
100% *Ch.r.* cw 15	364 ± 4	67 ± 2	0	0.9937	12.76	4.3
100% OMSW	388 ± 4	136 ± 8	0	0.9825	21.49	6.5
85% OMSW–15% *Ch.r.* 6145	298 ± 2	139 ± 5	0	0.9938	9.58	2.9
85% OMSW–15% *Ch.r.* cw 15	295 ± 6	133 ± 16	0	0.9404	29.67	13.4
75% OMSW–25% *Ch.r.* 6145	404 ± 7	91 ± 7	0	0.9722	28.33	3.8
75% OMSW–25% *Ch.r.* cw 15	295 ± 6	101 ± 10	0	0.9514	26.99	13.4
50% OMSW–50% *Ch.r.* 6145	529 ± 4	129 ± 3	0	0.9962	14.14	2.3
50% OMSW–50% *Ch.r.* cw 15	438 ± 4	88 ± 3	0	0.9943	14.50	2.8

* *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15, B_{max}: ultimate methane production, R_{max}: maximum methane production rate, S.E.E.: Standard error of estimate, λ: calculated lag times

**Error $((B_{max\ experimental} - B_{max\ model})/B_{max\ experimental})\cdot 100$

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Chapter 6

Evolution of control parameters in biochemical methane potential test for olive mills solid waste (OMSW), soft-hydrothermal pre-treated OMSW and the co-digestion mixture of OMSW and microalga *Dunaliella salina*

This chapter to be submitted as:

M.J. Fernández-Rodríguez; D. De la Lama-Calvente; A. Jiménez-Rodríguez;R. Borja; B. Rincón-Llorente. Evolution of control parameters in biochemical methane potential test for olive mills solid waste (OMSW), soft-hydrothermal pre-treated OMSW and the co-digestion mixture of OMSW and microalga *Dunaliella salina*

Abstract

The aim of the present work was to compare the evolution of control parameters in the mesophilic anaerobic digestion of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW) and a co-digestion mixture of 95% OMSW and 5% microalga *Dunaliella salina*. The pH values remained relatively constant in the three experiments. During the co-digestion experiment, the contribution of the microalga helped to smooth the hydrolytic stage and made it more spaced over time, improving the AD process. The volatile fatty acid accumulation decreased in comparison with that obtained for OMSW and SHP OMSW, reducing the slight inhibition observed during the OMSW and SHP OMSW experiment. Final values of methane yield of 380±1 mL CH_4/g VS_{added} for the OMSW, 424±2 mL CH_4/g VS_{added} for the SHP OMSW and 491±1 mL CH_4/g VS_{added} for the co-digestion mixture were determined. Two mathematical models, first-order kinetics and modified Gompertz model were employed to fit the experimental data with the aim of elucidating the anaerobic biodegradation and obtain the kinetic constants in the three cases studied. Both models allowed for adequately fitting the experimental results of methane production with time in the anaerobic experiments. The kinetic constant, *k*, of the first-order model increased by 12% when the OMSW was co-digested with *Dunaliella salin*a (95% OMSW-5% *D. salina*) compared with the

values achieved for untreated OMSW and thermally pretreated OMSW. The modified Gompertz model revealed that the maximum methane production rate, R_m, for co-digestion of 95% OMSW-5% *D. salina* and thermally pre-treated OMSW increased by 34.7% and 10.3% compared to the value obtained for raw OMSW.

1. Introduction

The valorization of waste is the most viable alternative to reduce the large amount of waste generated in agriculture and the agro-industrial sector every year. This recycling strategy not only avoids disposal cost and environmental issues, but also brings a valorization pattern to the agricultural and agro-industrial sectors (Muchagato et al., 2018). The valorization of by-products is a trend that is in progress and has been increasing its value in the last decade (Borges et al., 2019, Meini et al., 2019). The olive oil industry is one of the main agro-economic activities in Spain and this is a sector that is booming worldwide. More and more countries are starting to produce olive oil, although the Mediterranean area contributes more than 80% to the worldwide production. Spain and Italy are the two leaders in olive oil production, while Greece holds the third place (Carbone et al., 2018).

During the 90s, a new extraction system for olive oil, the two-phase system, expanded rapidly in Spain. This innovative system contributed to an improvement in the quality of the product and a decrease in water consumption during the process (del Pozo et al., 2018). Even so, during two-phase olive oil processing a huge amount of highly polluting waste (800 kg of olive mill solid waste for one ton of processed olives) is generated in a short period of time (November-February) every year. The olive mill solid waste

(OMSW) is underutilized and often ends up polluting the environment (del Pozo et al., 2018). OMSW is a lignocellulosic by-product with high water content and it is characterized as a highly polluting waste, not only because of the large amount that is generated every year but also due to its composition. OMSW is characterized as a by-product with acidic pH and high electrical conductivity which is very rich in organic matter since it has a high content of sugars, polyalcohols, pectins, lipids and aromatic compounds which give it a phytotoxic and antimicrobial character (Muktadirul Bari Chowdhury et al., 2013).

Anaerobic digestion (AD) is a biological process that breaks down organic materials in the absence of oxygen (anaerobic conditions) into methane and carbon dioxide, mainly. The AD of OMSW is a widely studied process (Borja et al., 2002, Christoforou and Fokaides, 2016; Rincón et al., 2007). There is also information about the use of different pre-treatments on OMSW or lignocellulosic wastes before AD where methane production has been improved: thermal pre-treatments on OMSW (Rincón et al., 2013), ultrasound pre-treatments (Rincón et al., 2014) or microwave pre-treatments (Rincón et al., 2016). Another widely studied alternative to improve methane yield during the AD of OMSW is co-digestion with microalgae or with nitrogen-rich co-substrates that help to balance the carbon/nitrogen (C/N) ratio and

therefore obtain an improvement in biogas production (Fernández-Rodríguez et al., 2014).

The biological methane potential (BMP) test has proved to be a suitable method to obtain maximum methane production and provide valuable information for optimizing the design and functioning of an anaerobic digester (Holliger et al., 2016). Literature related to the BMP assays is extensive, and shows that this test has been used to evaluate a wide variety of substrates. However, there is scarce information and very few reports on the evolution or temporal variation in the parameters of a BMP assay. This type of information can help improve the implementation of a full scale AD plant as well as provide important information about the evolution of the AD process.

This work aims to give information about the evolution of parameters during the BMP test of raw OMSW, soft hydrothermal pretreated OMSW (SHP OMSW) and co-digestion of the best percentage mixture OMSW-microalga *Dunaliella salina.* The parameters soluble chemical oxygen demand (sCOD), pH, alkalinity, ammonium, volatile fatty acids (VFA) and methane production were evaluated over time. At present, no previous available literature has reported on the comparative evolution of different parameters during the AD of this raw substrate, its AD previously thermally pre-treated (SHP OMSW) or a co-digestion mixture of the same substrate with microalga *Dunaliella salina*.

2. Materials and methods

2.1 Substrates and inoculum

Three different substrates were used: (i) OMSW, (ii) OMSW, pre-treated with a soft hydrothermal pre-treatment at 121ºC, 30 minutes 1.1 bar (SHP OMSW) and (iii) the co-digestion mixture of 95% OMSW and 5% microalga *Dunaliella salina* (95% OMSW-5% *D. salina*). The pre-treatment at 121 ºC and 1.1 bar during 30 min was chosen based on previous results obtained from different soft hydrothermal pre-treatments carried out on OMSW (unpublished data).

500g of OMSW were introduced into a 1-L autoclaved bottle and then it was autoclaved under the conditions selected. The sample was then cooled to room temperature and stored at 4 ºC for less than 24 h until use. The co-digestion mixture assayed was the best percentage mixture obtained in previous studies. Table 1 shows the main characteristics of the substrates and the inoculum used during the experiments.

OMSW was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). Olive stone pieces were removed using a 2 mm mesh. *Dunaliella salin*a (*D. salina*) was provided as a lyophilized

biomass by Huelva University, Huelva (Spain). The main characteristics of the *D. salina* are presented in Table 1. Anaerobic reactors were inoculated with biomass obtained from an industrial up-flow anaerobic sludge blanket reactor for wastewater from a brewery located in Seville (Spain).

2.2 Biochemical methane potential (BMP) tests

The tests were carried out in a multi-batch reactor system with an effective volume of 200 mL. The reactors were continuously agitated by magnetic bars at 440 rpm and placed in a thermostatic water bath at mesophilic temperature (35 ± 2 ºC).

The inoculum to substrate ratio was 2 (VS basis). Each reactor containing 150 mL of inoculum and the amount of substrate needed to give the required inoculum to substrate ratio. Finally, 150 μL of a trace element solution were added.

2.2 Biochemical methane potential (BMP) tests

The tests were carried out in a multi-batch reactor system with an effective volume of 200 mL. The reactors were continuously agitated by magnetic bars at 440 rpm and placed in a thermostatic water bath at mesophilic temperature (35±2ºC).

Table 1. Characteristics of the inoculum, olive mill solid waste (OMSW), and microalga *Dunaliella salina* used in the experiments. Where TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, sCOD: soluble chemical oxygen demand, TKN: Total Kjeldahl nitrogen, TA: total alkalinity, nd: not determined.

Parameters	**Values Inoculum**	**Values OMSW***	**Values D. salina****
TS (g/kg)	27.7±1.8	231.5 ± 2.3	887.9 ± 7.3
VS (g/kg)	20.9±1.7	201.8 ± 2.6	472.3 ± 8.1
COD (g O_2/kg)	nd	325.1 ± 0.4	272 ± 8
sCOD (g O_2/kg)	nd	144.4 ± 4.1	nd
TKN (g/kg)	nd	nd	7. 9± 0.7
pH	7.1±0.2	4.7 ± 0.1	8.22±0.2 (1:20)***
TA (g $CaCO_3$/kg)	nd	2.7 ± 0.0	nd

*Concentrations expressed as: weight/weight of wet sample. **Concentrations expressed as: weight/weight of lyophilized sample. *** (w:v) using distilled water.

The inoculum to substrate ratio was 2 (VS basis). Each reactor containing 150 mL of inoculum and the amount of substrate needed to give the required inoculum to substrate ratio. Finally, 150 μL of a trace element solution were added.

The composition of the trace element solution was: $FeCl_2 \cdot 4H_2O$, 2000 mg/L; $CoCl_2 \cdot 6H_2O$, 2000 mg/L; $MnCl_2 \cdot 4H_2O$, 500 mg/L; $AlCl_3 \cdot 6H_2O$, 90 mg/L; $(NH_4)_6Mo_7O_2 \cdot 4H_2O$, 50 mg/L; H_3BO_3, 50 mg/L; $ZnCl_2$, 50 mg/L; $CuCl_2 \cdot 2H_2O$, 38 mg/L, $NiCl_2 \cdot 6H_2O$, 50 mg/L, $Na_2SeO_3 \cdot 5H_2O$ 194 mg/L and EDTA 1000 mg/L. Reactors with inoculum and trace element solution but without substrate addition were used as controls.

The reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 3N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. The biogas volume was expressed at standard pressure and temperature conditions (273K, 1bar). The AD experiments were run for a period of c.a. 34 days until the accumulated gas production remained essentially unchanged. 10 reactors of each experiment were placed and 7 of them were sacrificed for different analyses in order to evaluate the evolution of the different parameters.

2.3 Analytical methods

All analyses were performed according to the Standard Methods of APHA (APHA, 1998). The following parameters were measured: total chemical oxygen demand (COD), soluble chemical oxygen demand (sCOD), total solids (TS), volatile solids (VS), Total alkalinity (TA), pH, Total Kjeldahl Nitrogen (TKN) and volatile fatty acids (VFA). Soluble parameters were determined after sample centrifugation (Eppendorf, 10000 rpm, 10 min) and filtration (glass fiber filter 47 mm).

TS and VS were determined according to the standard methods 2540B and 2540E, respectively (APHA, 1998); COD was determined by the method described in detail elsewhere (Raposo et al., 2008), while sCOD was determined using the closed digestion and the colorimetric standard method 5220D (APHA, 1998). pH was determined using a pH-meter model Crison 20 Basic. TA was determined by pH titration to 4.3 (APHA, 1998). TKN was determined using a method based on the 4500-Norg B of Standard Methods (APHA, 1998).

Individual VFA from C2 to C7 including iso-C4, iso-C5 and iso-C6 were analyzed using a Gas Chromatograph (Shimadzu GC 2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol (polyethylene glycol modified by nitroterephthalic acid). Prior to injection, 900 μL of the sample

were mixed with 150 µL of H_3PO_4 (1:2, V:V) to adjust the pH to below 2.0 and 150 µL of a solution of crotonic acid (2000 mg/L) as internal standard. This mixture was centrifuged to remove any solids and transferred to a 1500 µL gas chromatography vial; the sample injection volume was 1 µL. The temperatures of the injector and detector were maintained at 200 ºC and 250 ºC, respectively, while the column temperature was increased from 120 to 160 ºC at an increasing rate of 10 ºC/min.

3. Results and discussion

Table 2 shows the pH evolution of the three experiments. The pH trend did not show relevant fluctuations in any of the experiments. During the AD of OMSW, the minimum pH value was 7.6 at the beginning of the experiment and the maximum value, 8.1, was reached at the end of the experiment. When the AD of SHP OMSW and co-digestion were carried out, the pH values were between 7.5 and 8. These values remained relatively constant in all the runs within the range described as optimal for the growth of methanogenic archaea (Yuan et al., 2016).

The evolution of the TA values throughout the OMSW experiment ranged from 4349±66 mg $CaCO_3$/L to 5551±62 mg $CaCO_3$/L, during SHP OMSW experiment TA values ranged from 3341 ±74 mg $CaCO_3$/L to 5534±67 mg $CaCO_3$/L and from

4182±68 mg $CaCO_3$/L to 5693±82 mg $CaCO_3$/L for the co-digestion experiment. In all three cases, the increase in alkalinity in the system was progressive, reaching the highest TA value at the end of the experiment. This increase in the TA values coincides with the ammonium accumulation in the system due to the degradation and stabilization of the organic matter that is being hydrolyzed. During the hydrolysis stage of the AD process, the complex organic matter was degraded into simpler soluble molecules that can be used as substrates for microbial metabolism by hydrolysis and acidogenesis. Then the substrates were consumed in the acetogenesis and methanogenesis phases (Mao et al., 2019). Throughout the three experiments, the final ammonium concentration was below the limit established as toxic for the evolution of the AD process (Polizzi et al. 2018). The TA values were also relatively stable, and within the optimal range for the AD process, indicating good process stability and self-buffering capacity during the three experiments carried out (Fannin et al., 1987).

Table 2. Individual volatile fatty acids, total volatile fatty acids (total VFA), pH and total alkalinity (TA) evolution in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*.

Substrate	Day	Acetic Acid (mg/L)	Iso-butyric Acid (mg/L)	Butyric Acid (mg/L)	Valeric Acid (mg/L)	Caproic acid (mg/L)	Total VFA (mg/L)	pH	TA (mg $CaCO_3$/L)
OMSW	2	NF	422	NF	125	NF	547	7.59	4349±13
	4	NF	762	NF	238	NF	1000	7.81	4481±58
	6	1710	825	NF	448	251	3226	7.81	4364±02
	8	NF	NF	NF	NF	NF	NF	7.79	4528±71
	11	NF	NF	NF	NF	NF	NF	7.88	4040±54
	14	NF	NF	NF	NF	NF	NF	7.86	4216±47
	21	NF	NF	NF	NF	NF	NF	8.08	5551±00
	34	NF	NF	NF	NF	NF	NF	8.32	5190±16
Co-digestion	2	NF	402	NF	NF	NF	402	7.49	4495±12
mixture (95%	4	NF	NF	NF	NF	NF	NF	7.68	4182±61
OMSW-5%	6	NF	460	NF	NF	747	1027	7.63	4370±28
Dunaliella	8	NF	578	NF	NF	421	999	7.77	4818±11
salina)	11	NF	NF	NF	NF	NF	NF	7.89	4812±95
	14	NF	NF	NF	NF	NF	NF	7.89	4799±21
	21	NF	NF	NF	NF	NF	NF	8.13	5148±52
	34	NF	NF	NF	NF	NF	NF	8.36	5693±20
SHP OMSW	2	NF	318	NF	NF	NF	318	7.46	3828±21
	4	NF	625	477	466	484	2052	7.71	4608±17
	6	NF	NF	NF	NF	NF	NF	7.59	3341±46

	8	NF	NF	NF	NF	NF	NF	7.71	4595±34
	11	NF	NF	NF	NF	NF	NF	7.54	4998±47
	14	NF	NF	NF	NF	NF	NF	7.82	4609±65
	21	NF	NF	NF	NF	NF	NF	7.94	4986±30
	34	NF	NF	NF	NF	NF	NF	8.30	5534±76

*NF: not founded

VFA content is one of the most widely used parameter to control the stability of the AD process. VFA concentration is the main factor affecting the intermediate alkalinity (Yu et al., 2018). The variation in VFA throughout time is summarized in Table 2. Throughout the SHP OMSW experiment, the VFA concentration was at its highest on day 4. The concentration of iso-butyric acid, butyric acid, valeric acid and caproic acid in the SHP OMSW experiment reached values of 625 mg/L, 477mg/L, 466 mg/L and 484 mg/L, respectively. The total VFA concentration reached on day 4 was 2052 mg/L. The pre-treatment accelerated the solubilization (hydrolysis) of OMSW and reduced the particle size so that a shortened hydrolytic stage could be observed. The total VFA concentrations observed during the AD of OMSW were significantly higher than those obtained for the SHP OMSW experiments. The OMSW VFA accumulation started on day 2 and was at its highest on day 6. Then the VFA concentration decreased until 0 on day 8. The maximum VFA peak was observed two days later than that observed during the SHP OMSW AD. The highest VFA accumulation during OMSW AD was on day 6 and mainly consisted of acetic acid (1710 mg/L), Iso-butyric acid (825 mg/L), valeric acid (448 mg/L) and caproic acid (251 mg/L), reaching a total VFA concentration of 3226 mg/L. Hydrolytic bacteria took longer to decompose the untreated or raw OMSW into simpler substances, and a large accumulation of VFA was observed on the 6^{th} day of the experiment, indicating that the AD process is not as

direct as in the case of pre-treatment. Both SHP OMSW and OMSW AD produced a VFA accumulation above the limit established by Siegert and Banks (2005). Siegert and Banks (2005) reported the inhibition of cellulolytic activity and, therefore, in the rate of cellulose hydrolysis when the VFA concentration was equal to or greater than 2 g/L, regardless of the system pH. During the co-digestion experiment, although most of the organic matter came from the OMSW, the contribution of the microalgae helped to smooth the hydrolytic stage, making it more evenly-spaced over time, thus improving the AD process. The VFA accumulation decreased in comparison with that obtained for OMSW. The main VFA concentrations were caproic acid (747 mg/L), iso-butyric acid (578 mg/L) and valeric acid (421 mg/L) on day 6, but similar values were reached on day 8 (iso-butyric acid=460 mg/L and caproic acid=747 mg/L). The VFA accumulation started on day 2 and was observed until day 8, but the total VFA maximum concentration was lower than those observed during the OMSW and SHP OMSW experiments and did not exceed the limit established by Siegert and Banks (2005) as toxic.

Figure 1 shows the evolution of sCOD throughout the trials. The organic matter solubilization was quite different throughout the three experiments. In the co-digestion experiment, a constant concentration of soluble organic matter was observed. The contribution of microalgae to the system added a more easily

biodegradable organic matter than the lignocellulosic OMSW, maintaining the values of sCOD almost constant at around 5000 mg O_2/L. Only a decrease in the soluble organic matter concentration was observed between days 8 and 10, in which, sCOD values of 3864 mg O_2/L and 4328 mg O_2/L were reached, respectively (Figure 1). This constant concentration of dissolved organic matter led to a synergism between the nutrients and bacteria involved in the anaerobic digestion process. During the co-digestion of water hyacinth and banana peels, Barua et al. (2019) observed a synergism that balanced the nutrients and the existence of adaptable and dynamic microbial community. The sCOD concentration observed during co-digestion was higher than those observed in the SHP OMSW and OMSW AD experiments. Similar results were reported by Yin et al. (2016) for co-digested activated sludge and food waste. They observed that sCOD was higher in the co-digestion than in the mono-digestion of substrate with and without pre-treatment. The sCOD concentration in the OMSW and SHP OMSW experiments often fluctuates considerably. The initial sCOD value in the OMSW experiment was 2203 mg O_2/L, and 2771 mg O_2/L for the SHP OMSW experiment. This small difference is due to the effect of the thermal pre-treatment on the substrate, which helped to break the lignocellulose fibers and solubilized organic matter (Pagliaccia et al., 2019).

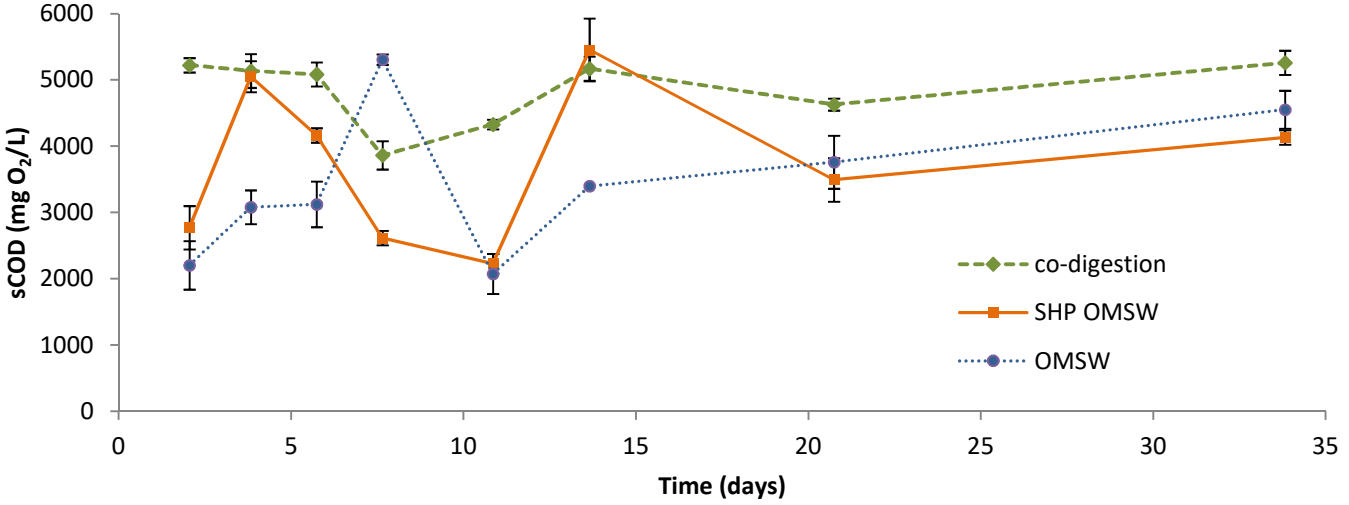


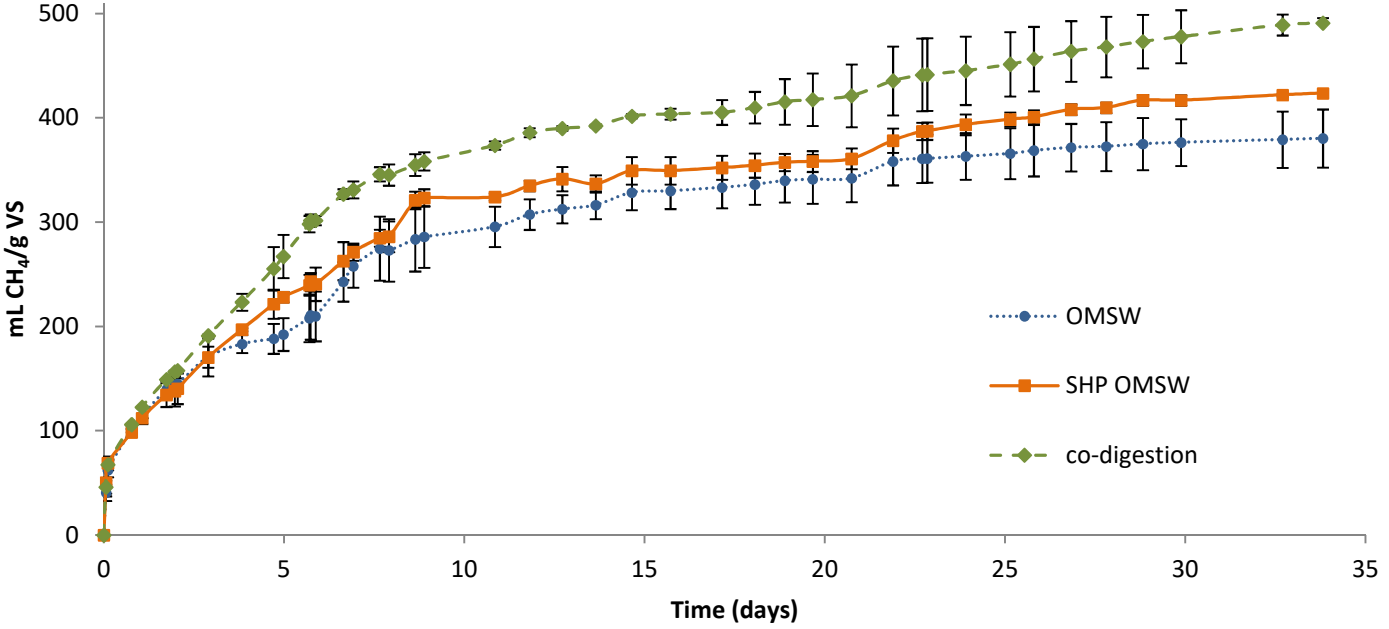
Figure 1. Soluble Chemical oxygen demand (sCOD) evolution in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*.

The effect of thermal pre-treatment was also observed over time, since in the SHP OMSW experiment two maximum peaks of organic matter solubilization were observed, the first one on day 2 and the second one on day 14 after the start of the experiment. On the other hand, during the AD of the OMSW, only a maximum peak of organic matter solubilization was observed and it was delayed over time, since it was observed on day 8. These fluctuations in organic matter solubilization did not allow a synergism between nutrients and the bacteria community. During OMSW AD hydrolysis was observed to be the rate-limiting step. The hydrolysis step is the main bottleneck during a lignocellulose substrate AD process due to the low biodegradability of the substrate (Chiu and Lo, 2016). Thermal pre-treatment helped to break cellulose, hemicellulose and lignin fibers (Rincón et al., 2013) but it was still the hydrolytic bacteria that caused an imbalance in the AD process. However, during co-digestion, an improvement in the hydrolysis step was observed, with the co-substrate acting as a catalyst for hydrolytic enzymes and a synergism was observed to balance the nutrients with the bacterial community. Several authors have observed an improvement in enzymatic activity during the anaerobic co-digestion of organic waste and microalgae (Chiu and Lo 2016).

The effect of pre-treatment (SHP OMSW) and anaerobic co-digestion of OMSW and the microalgae *D. salina* in a mixing ratio

of 95 and 5%, respectively, were investigated in comparison with the AD of raw OMSW. The methane yields for the three experiments are shown in Figure 2. After 34 days of AD the cumulative methane production showed the same variation tendency in each experiment. The methane production in the entire test grew exponentially in the first 8 days and then it was stabilized. Despite being a difficult biodegradable substrate, the methane production was immediate in each of the three experiments, possibly because the bacteria used an inner fraction that was easily degradable, thus, the hydrolysis step might have begun with this more available substrate (Cirne et al., 2007). In all three cases the methane yield became stable from the 22nd day after the experiment had started. Finally, higher methane productions were observed for the co-digestion than for individual substrates, OMSW and SHP OMSW. Final values of 380±1 mL CH_4/g VS_{added} for the OMSW, 424±2 mL CH_4/g VS_{added} for the SHP OMSW and 491±1 mL CH_4/g VS_{added} for the co-digestion mixture were determined. A 21 and 4% increase in methane yield was achieved for co-digestion and SHP OMSW compared to the raw OMSW, respectively.

Figure 2. Methane yield obtained in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*.



The methane yield increase obtained in the SHP OMSW compared to raw OMSW (4%) was similar to the one reported by Rincón et al. (2013) for the BMP test of thermally pre-treated OMSW compared to the raw OMSW (5%). Rincón et al. (2013) reported a methane yield of 373 ±4 mL CH_4/g VS_{added} for the BMP test of raw OMSW and 392±14 mL CH_4/g VS_{added} for the BMP test of pre-treated OMSW at 120 °C for 180 min.

The maximum daily methane production of 48±8 mL CH_4/g VS_{added} was achieved on the 7th day for the OMSW BMP test and 58±2 and 76±5 mL CH_4/g VS_{added} on day 5 for the SHP OMSW and co-digestion mixture, respectively. Fernández-Rodríguez et al. (2014) also obtained an improvement in methane production through the co-digestion OMSW-microalga *Dunaliella salina*. However, a lower methane yield was achieved with the mixture 75% OMSW-25% *D. salina* (330mL CH_4/g VS_{added}), improving OMSW methane production by only 2.8% (Fernández-Rodríguez et al., 2014).

3.1 Estimation of model parameters by kinetic modeling

3.1.1 First-order kinetic model

In order to study the process kinetics and estimate the process performance in the AD and co-digestion of the three cases studied, the following first-order kinetic model was used:

$$G = G_m \cdot [1 - \exp(-k \cdot t)] \quad (1)$$

where G is the cumulative specific methane production (mL CH_4/g VS_{added}), G_m is the ultimate methane production (mL CH_4/g VS_{added}), k is the specific rate constant ($days^{-1}$) and t is the digestion time (days). This kinetic model is normally applied to assess the kinetics of the batch AD processes for different types of biodegradable substrates (Li et al., 2012). This model is based on the assumption that methane production is proportional to the amount of substrate and not limited by microbial cell mass (Wang et al., 2017).

Table 3 summarizes the kinetic parameters obtained from Eq. (1) for the co-digestion mixture and the two substrates studied

alone. Values situated after ± represent the standard deviation of each parameter. As can be seen, deviations between the experimental G_m values (Figure 2) and the theoretical ones (Table 3) lower than 7.7% were obtained for all the cases studied. In addition, the low values of the standard deviations and the high determination coefficient values prove the appropriate fit of the experimental results to the proposed model.

As can be seen in Table 3, the ultimate methane production increased by 8.5% and 24% when the OMSW was thermally-pre-treated and co-digested with *D. salina* (95% OMSW-5% *D. salina*) respectively, compared to the value obtained for untreated OMSW. It was reported that BMP tests of mixtures of source selected OFMSW (organic fraction of municipal solid wastes) and sewage sludge showed a methane production increase from 18% to 47% compared to the AD of single sewage sludge (Cabbai et al., 2013). The kinetic constant, *k*, increased by 12% when the OMSW was co-digested with *D. salina* (95% OMSW-5% *D. salina*) compared with the values achieved for untreated OMSW and thermally pretreated OMSW (0.17 $days^{-1}$ in both cases). In the same way, no significant differences were observed by Donoso-Bravo et al. (2010) in the kinetic constant of this first-order model during batch

Table 3. Kinetic parameters obtained from the first-order kinetic model in the batch anaerobic digestion experiments of OMSW, soft hydrothermal pre-treated (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*.

Substrate	G_m **(mL CH_4/g VS_{added})**	k **($days^{-1}$)**	***R^2**	****S.E.E.**	*****Error*** **(%)**
OMSW	364 ± 6	0.17 ± 0.01	0.9778	22.09	4.1
SHP OMSW	395 ± 6	0.17 ± 0.00	0.9811	22.42	6.3
Co-digestion 95%OMSW-5% *Dunaliella salina*	451 ± 5	0.19 ± 0.00	0.9862	22.21	7.7

*R^2: coefficient of determination **S.E.E.: Standard error of estimate; ***Error was defined as the difference between measured and predicted methane yield values.

AD tests of untreated secondary sludge (from an urban wastewater treatment plant) and thermally pre-treated secondary sludge at 175 ºC for 30 minutes (0.22 and 0.18 $days^{-1}$, respectively). By contrast, a reduction of around 50% was observed in the first-order kinetic constants of BMP tests of wheat straw (from 0.10 to 0.05 $days^{-1}$) and sugarcane bagasse (from 0.083 to 0.048 $days^{-1}$) when these wastes were subjected to an autoclaving pre-treatment (at 121 ºC, 60 minutes) compared to their respective untreated wastes (Bolado-Rodríguez et al., 2016). This decrease was attributed to the presence of inhibitory compounds for AD after thermal pre-treatment. On the other hand, the co-digestion of a hardly biodegradable substrate, i.e. sugar bagasse (SB), and easily biodegradable waste, i.e. fruit vegetable waste (FVW), at a ratio 70% FVW-30% SB allowed for increasing the kinetic constant from 0.0023 $days^{-1}$ (100% SB) to 0.022 $days^{-1}$ (70% FVW-30% SB) (Vats et al., 2019). This increase is much higher than that observed in the present work when the OMSW (k=0.17 $days^{-1,}$ for 100% OMSW) was co-digested with *D. salina* at a ratio (95% OMSW-5% *D. salina*) (k=0.19 $days^{-1}$). By contrast, the co-digestion of *Salvinia molesta* (SM), one of the free-floating aquatic weeds (that can grow very rapidly) with rice straw (RS) at different mixture ratios (from 40:60 to 0:100, SM:RS) at different initial pH values (6-8) gave the same first-order kinetic constant values (0.01 $days^{-1}$) regardless of the concentration of SM and RS in the mixture and initial pH (Syaichurrozi et al., 2018).

3.1.2 Modified Gompertz Kinetic Model

On the other hand, the Modified Gompertz Kinetic Model is a sigmoid function that is considered as a type of mathematical model for a time series (Amiri et al., 2017). Therefore, it can be the one of the best functions for predicting the biogas production in a batch-mode AD process. Many researchers have studied the application of different kinetic models and found that the Modified Gompertz model has one of the best fit to the data from biogas or methane production as a function of time under anaerobic processes conducted in batch mode. In addition, the Modified Gompertz model was calibrated and examined using many experimental data (Amiri et al., 2017; Donoso-Bravo et al., 2010; Donoso-Bravo et al., 2016; Li et al., 2011).

In the Modified Gompertz model, the cumulative methane production is related to the digestion time through the following equation:

$$B = B_m * \exp\left[-\exp\left[(R_m * e/B_m) * (\lambda - t) + 1\right]\right] \qquad (2)$$

where B is the cumulative methane production at time t (mL CH_4/g VS_{added}); B_m is the maximum methane production or methane yield potential (mL CH_4/g VS_{added}); R_m is maximum methane production rate (mL CH_4/g VS_{added} ·d); λ is the lag time (d); t is the digestion time (d) at which the cumulative methane production is calculated; and finally, e is the exp(1) = 2.7183. The parameters B_m, R_m and λ were calculated for each one of the runs studied using the non-linear regression approach with the software Sigma Plot 11.0. Table 4 shows the values for the model parameters obtained from the Modified Gompertz model for the three substrates assayed. Similarly to what occurred with the experimental maximum methane production values, the theoretical maximum methane production was 8 and 23% higher when the OMSW was thermally pre-treated and when it is co-digested with *D. salina* compared to the value obtained for untreated OMSW. Therefore, a considerable increase in the biodegradability of the substrate was observed when the OMSW was thermally pre-treated or co-digested with *D. salina*, especially in this last case, compared to the raw OMSW.

In addition, the differences between measured and predicted methane yields were found to be only 6.7, 10.1 and 12.1% for the untreated OMSW, thermally pre-treated OMSW and OMSW co-digested with *D. salina*, respectively.

Table 4. Parameters of the Modified Gompertz model for the three substrates studied olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*.

Substrate	B_m **(mL CH_4/g VS)**	R_m **(mL CH_4/g VS d)**	λ **(d)**	***R^2**	****S.E.E.**	*****Error (%)**
OMSW	355 ± 6	40.6 ± 2.1	$8 \cdot 10^{-4}$	0.9631	28.73	6.7
SHP OMSW	383 ± 6	44.9 ± 2.7	$6 \cdot 10^{-11}$	0.9688	29.11	10.1
Co-digestion 95%OMSW-5%*Dunaliella salina*	437 ± 7	54.7 ± 4.1	$1 \cdot 10^{-8}$	0.9751	30.16	12.1

*R^2: coefficient of determination **S.E.E.: Standard error of estimate; ***Error was defined as the difference between measured and predicted methane yield values.

The high values for the determination coefficients (R^2) and the low values of the standard errors of estimates (Table 4), again show the excellent fit of the experimental results to the Modified Gompertz Model.

The maximum methane production rate, R_m, for the co-digestion of 95% OMSW-5% *D. salina* and thermally pre-treated OMSW increased by 34.7 and 10.3% compared to the values obtained for the raw OMSW. Therefore, the thermal pre-treatment and co-digestion of this substrate with *D. salina* improved the rate of the AD process of OMSW, accelerating the methane production rate. In this way, Donoso-Bravo et al. (2016) found that the maximum methane production rate of untreated OMSW and thermally pre-treated OMSW at 148 ºC for 30 minutes were 28.1 and 42.7 mL CH_4/(g VS·d), respectively, showing an increase of 51% when the substrate was pre-treated. In any case, the R_m values achieved in the present work were always higher than those reported by Donoso-Bravo et al. (2016). In the same way, the modified Gompertz model applied to the batch AD of the microalga *Chlorella sp.* (grown in digested swine waste) and to thermally pre-treated *Chlorella sp.* revealed an increase in the R_m values from 30.5 to 37.5 and to 44.5 L CH_4/(g VS·d) when the temperature of pre-treatment increased from 70 to 90 and 120 ºC, respectively (Wang et al., 2015). These values were always higher than those

obtained for untreated *Chlorella* sp. (25.1 mL CH_4/(g VS·d) (Wang et al., 2015).

On the other hand, the R_m values found in the present work either for untreated OMSW or a mixture of 95% OMSW-5% *D. salina* were always much higher than those found during the anaerobic co-digestion of 40% *Salvinia molesta* and 60% rice straw (2.1-2.6 mL CH_4/(g VS· d)) regardless of the initial pH of the mixture (6-8) (Syaichurrozi et al., 2018).

Similarly to what occured in the present work, the anaerobic co-digestion of mixed microalgae (*Scenedesmus* sp. and *Chlorella* sp.) (MA) – food waste (FW) at a ratio of 20% MA-80% FW showed a R_m value of 44.3 mL CH_4/(g VS·d), which was 15% higher than that obtained for 100% FW (38.6 mL CH_4/(g VS·d)) and 266% higher than that achieved for 100% MA (12.1 mL CH_4/(g VS·d)) (Zhen et al., 2016).

4. Conclusions

The performance of AD of OMSW, thermally pre-treated OMSW and the co-digestion of 95% OMSW-5% *D. salina* were evaluated in this research and the corresponding biological degradations and kinetics of the processes were analyzed through model simulation. The introduction of the microalga *D. salina*

improved OMSW digestion performance, maintaining relatively constant soluble organic matter concentrations, reducing the VFA accumulation. The co-digestion reached the highest methane yield of 491 ± 22 mL CH_4/g VS at a ratio of 95% OMSW-5% *D. salina*, achieving 1.29-fold increase in comparison to that of the single OMSW (380 ± 28 mL CH_4/g VS). The first-order and the modified Gompertz models showed a good fit to the experimental results in the three scenarios studied, and, thus, could describe the kinetics of the digestion of OMSW, thermally pre-treated OMSW and the co-digestion of 95% OMSW-5% *D. salina*. The highest values for the kinetic constant (k) and the maximum methane production rate (R_m) achieved in the co-digestion of 95% OMSW-5% *D. salina* revealed the robustness of the co-digestion of this mixture compared to the single digestion of raw OMSW or thermally pre-treated OMSW.

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Chapter 7

Towards olive oil industry sustainability: Microalgae for the treatment of olive oil effluents and the anaerobic co-digestion of this biomass with olive mill solid waste.

This chapter to be submitted as:

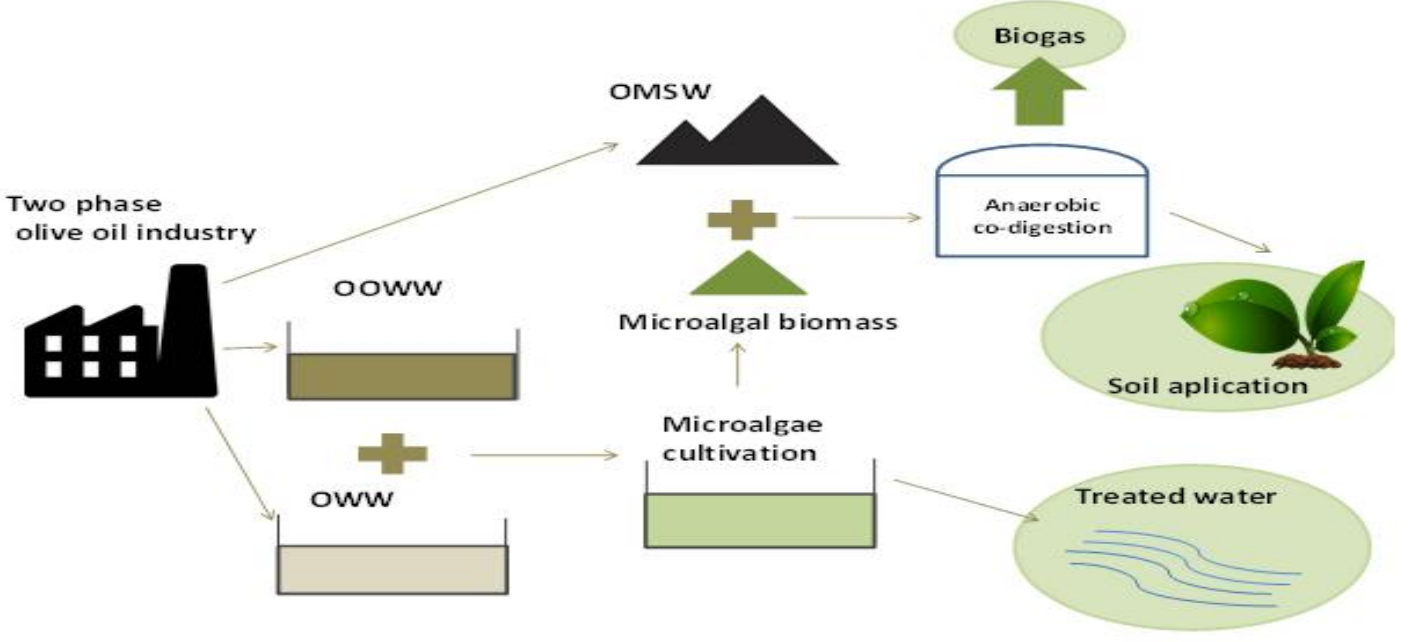
M.J. Fernández-Rodríguez; D. De la Lama-Calvente; M. García-González; J. Moreno-Fernández;A. Jiménez-Rodríguez; R. Borja; B. Rincón. Towards olive oil industry sustainability: Microalgae for the treatment of olive oil effluents and the anaerobic co-digestion of this biomass with olive mill solid waste.

Abstract

This study evaluates an integral treatment of the wastes derived from the two-phase olive oil extraction process: olive washing waters (OWW), olive oil washing waters (OOWW) and olive mill solid waste (OMSW). The research brings the treatment and valorization of these wastes to sustainable levels in a closed-loop process. In a first step, the microalga *Raphidocelis subcapitata* (*R. subcapitata*) was grown using a mixture of OWW and OOWW as culture media, and its potential for biomas production and nutrient removal (phosphate, nitrate, etc.) was demonstrated. The maximum specific growth rate of this microalga in these wastewaters was 0.31 ± 0.02 $days^{-1}$. A pseudo-first-order kinetic model was applied to describe the temporal variation of nutrient concentrations in the wastewater. It was found that the rate of phosphate removal (1.30 ± 0.09 $days^{-1}$) was around six times higher than that obtained for nitrate (0.27 ± 0.02 $days^{-1}$), total sugars (0.23 ± 0.09 $days^{-1}$) and soluble chemical oxygen demand (0.17 ± 0.04 $days^{-1}$). In a second step, the microalgal biomass (*R. subcapitata*) grown was used as co-substrate with OMSW for methane production by anaerobic co-digestion. The anaerobic co-digestion of the mixture 75% OMSW – 25% *R. subcapitata* increased the methane yield 7.0% and 64.5% compared to the anaerobic digestion of 100% OMSW and 100% *R. subcapitata,* respectively, and to the other mixture percentages tested. Therefore, the highest biodegradability was found for the

co-digestion mixture 75% OMSW – 25% *R. subcapitata.* In addition, this co-digestion mixture had a higher synergic effect than the other co-digestion mixtures studied. However, the mixture 25% OMSW-75% *R. subcapitata* showed the highest specific rate constant and maximum methane production rate compared to the other mixture percentages tested.

Graphical abstract



1. Introduction

Olive oil consumption is increasing worldwide due to its beneficial health properties (Gavahian et al., 2019). This leads to an increase in olive oil production; not only in countries that have traditionally consumed olive oil, but also more and more countries have begun to produce it (Bicen and Malter, 2019) . The olive tree and its industry is one of the main agro-economic activities in Europe, where 95% of the total worldwide production comes from the Mediterranean countries. Spain is the first olive oil producer in the world with Andalusia being the region where the largest olive oil production is concentrated (Aparicio-Ruiz et al., 2019).

In the early nineties, a more sustainable olive oil extraction system was implemented, the two-phase olive oil production system. This system not only provided the industry with an improvement in the quality of the oil, but also decreased the use of water and the energy consumption during the process (Alburquerque et al., 2004). Even so, during the two-phase olive oil production process three types of wastes are produced, a semisolid waste with high humidity, generally called olive mill solid waste (OMSW) or “alperujo”, the effluent from cleaning the olive fruit before starting the production process or olive washing wastewater (OWW) and finally, the olive oil washing waters (OOWW) coming from the vertical centrifuge of olive oilscleaning (Alburquerque et al., 2004; Ochando-Pulido et al., 2013). The OMSW is a

lignocellulosic by-product characterized by acidic pH, and high organic matter content due to the presence of sugars, tannins, phenols, polyalcohols, pectins and lipids (Maragkaki et al., 2018). The OOWW and OWW are characterized by pH of 4.5 and 6.3, Chemical Oxygen Demand (COD) contents of 1 and 15 g O_2/L, total phenol contents of 2400 and 0.4 mg/L and electrical conductivity of 9.0 and 0.9 mS/cm, respectively (Borja et al., 2006; Ochando-Pulido et al., 2013).

On average, throughout the olive oil production period, between 10 and 15 m^3 of OOWW are produced each day and 1 m^3 day^{-1} of OWW as well as 800 kg of OMSW per 1000 kg of processed olives, generating more than 3.2 million tons of its main waste (OMSW) each year (Ochando-Pulido et al., 2013).

Despite the great economic impact generated by the olive oil industry in Europe, and the existence of regulations at the European level that regulate the management of waste (Directive 2008/98 / CEE 19 November, as amended by Directive 2018/851 / EU, 30 May), there is no European standard that establishes controls for the handling and treatment of solid and liquid waste from olive oil mills. Usually these by-products are treated as wastes, stored in evaporation ponds each season, where the OMSW is reconcentrated and fermented, creating risks of contaminating groundwater with organic loads, generation of bad odours, etc (Karaouzas, 2018) .

In 2015, the European Commission proposed a series of measures to adopt towards a Circular Economy defined as sustainable economies whose central axes are reduced, reused and recycled. The reuse of by-products to extend their life and to obtain valuable products is the central axis of the Circular Economy.One of the proposals of the Circular Economy is to reduce the environmental impact of the industry and provide environmental and societal benefits.

In many countries, nutrients in wastewater are required to be removed significantly in order to meet, or at least come close to the quality of surface water before the effluent discharge of wastewater from treatment plants. The advanced wastewater treatment process, e.g., membrane bioreactor and phosphorous adsorption columns require high costs and complicated operation and management (Liu et al., 2017). However, nutrients in the wastewater can be effectively removed by microalgal uptake. Thus, microalgal cultivation provides an alternative pathway for nutrient removal from wastewater. Improving microalgal growth and nutrient removal rates are two crucial research gaps that must be overcome for full-scale plant operation with the aim of achieving a circular economy in the industries.

Microalgal growth in wastewater may offer not only nutrient removal but also a source of nitrogen-rich biomass.

Anaerobic digestion (AD) is a key tool that supports the Circular Economy due to organic wastes being converted into energy (Abad

et al., 2019). OMSW is a lignocellulosic biomass with a high carbon/nitrogen (C/N) ratio, which could hinder its AD, giving low methane yields by lack of nitrogen. The optimum C/N ratio of a substrate for AD must be between 20:1 and 30:1 (Xu et al., 2017).The co-digestion of microalgae and OMSW would not only improve the C/N ratio of the substrate, but it would also improve the enzymatic activity of the bacteria and dilute the concentration of inhibitory substances in the reactor (Barua et al. 2019).

The purpose of this work was to evaluate a closed-loop process for the olive oil industry using microalgae as a wastewater treatment system for OOWW and OWW and then using that grown biomass in these wastewaters as co-substrate for the OMSW for biogas production with the aim of including the olive oil industry into a Circular Economy.

2. MATERIAL AND METHODS

2.1. OMSW, OOWW, OWW and the Inoculum for anaerobic digestion

The three by-products coming from the two-phase olive oil process (OMSW, OOWW and OWW) were collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). The OMSW was sifted through a 2 mm mesh with the purpose of removing olive stone pieces.

The inoculum was obtained from an industrial upflow anaerobic sludge blanket reactor which treats brewery wastewater located in Sevilla (Spain). The main characteristics of the by-products and the inoculum are shown in Table 1.

2.2. Microalgal cultivation

Raphidocelis subcapitata (R. subcapitata) was obtained from the culture collection of the Institute of Plant Biochemistry and Photosynthesis, IBVF (CSIC), (Seville, Spain).

Raphidocelis subcapitata is a synonym of *Pseudokircheniella subcapitata* (http//:www.algaebase.org). Although it is incorrect to treat *Raphidocelis subcapitata* as a synonym of *Selenastrum capricornutum*, the algae commonly represented in culture collections as *Selenastrum capricornutum* is not this species but *Raphidocelis subcapitata* (http//:www.algaebase.org). For this reason and due to lack of bibliography on *Raphidocelis subcapitata*, many of the data of this research have been compared with *Selenastrum capricornutum.*.Initially, *Raphidocelis subcapitata* was grown photoautotrophically on the medium described by Arnon et al. (1974) modified to contain 4 mM K_2HPO_4. Biomass was harvested and, finally separated by centrifugation for 10 min 2000×g and the seed was placed into OOWW diluted with OWW and $NaNO_3$ was added. Three reactors were inoculated from batch-grown cells and operated on batch mode for eight days.

Table 1. Characteristics of Olive Oil washing waters (OOWW), olive washing waters (OWW), olive mill solid waste (OMSW) and *Raphidocelis subcapitata* (*R. subcapitata*) used in the experiments. Where TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, C/N: ca$_t$bon/nitrogen, sCOD: soluble chemical oxygen demand and nd: not determined.

Parameter	**Values OOWW**	**Values OWW**	**Values OMSW**	**Values *R. subcapitata***	**Values Inoculum**
TS (g/L)	14.8 ± 5.4	4.4 ± 1.1	262.3 ± 1.7*	52.6 ± 0.9	33.8± 0.4
VS (g/L)	14.6 ± 2.5	2.6 ± 2.4	229.1 ± 2.0*	52.1 ± 0.6	27.2 ± 0.6
COD(g O_2/L)	19.4 ± 2.8	2.8 ± 1.5	354.1 ± 4.3**	nd	nd
sCOD(g O_2/L)	nd	nd	144.4 ± 4.2	nd	nd
pH	4.7 ± 0.1	10.2 ± 0.3	4.7 ± 0.1	nd	6.9 ± 0.2
C/N ratio	nd	nd	31.6± 0.3	8.8 ± 0.2	nd

* (g/ kg); **(g O_2/kg)

The growth was carried out in a 2 L capacity reactor at 25 ºC, pH with maintained at 7.5 and illuminated with white-light lamps (Phillips Master TL5 HO 24 W/840) following a solar daylight cycle (12 h light: 12 h dark) which provided maximal incident irradiance of 1500 $\mu Em^{-2} s^{-1}$ in the center of the reactor.

The growth of the microalgae was determined by chlorophyll measurement. One mL aliquots of cell suspension were centrifuged at 2000×g for 5 min, 1 mL methanol was added to the pellets containing the cells, and the pigments were extracted at 70 °C for 15 min. The methanolic extract was centrifuged at 2000×g for 5 min to remove cell debris. The chlorophyll content was measured spectrophotometrically at 650 and 665 nm.The main characteristics of microalga are shown in Table 1.

Wastewater samples were taken every day to determine nutrient removal from wastewater; sCOD, nitrate, phosphate and total sugar contents were analyzed.

2.3. Biological methane potential (BMP) assays

The experiment was carried out in batch in 250 mL reactors continuously agitated by magnetic bars at 440 rpm at mesophilic temperature. The BMP tests were operated with different blending ratios of OMSW and microalgae (100% OMSW, 75% OMSW-25% *R. subcapitata*, 50% OMSW-50% *R. subcapitata*, 25% OMSW-75% *R. subcapitata* and 100% *R. subcapitata*). The

inoculum to substrate ratio was 2 to 1 (VS basis). All the mixtures were run in triplicate and three blanks with only inoculum were used as control. A 1% trace element solution was added in a composition described by Fernández Rodríguez et al., (2014). The reactors were flushed with N_2 gas prior to starting the experiment. The produced biogas was passed through a 3N NaOH solution and only the methane production was recorded. The BMP test was carried out until the accumulated gas production remained essentially unchanged and the production was expressed using the normal temperature and pressure conditions.

2.4. Analytical methods

Phosphate (PO_4-P) and nitrate (NO_3-N) were measured with Hach Lange kits and a Hach Lange DR3900 spectrophotometer. Total solids (TS), Volatile solids (VS), soluble chemical oxygen demand (sCOD) and total alkalinity (TA) were determined in accordance with Standard methods (American Public Health et al., 2005). COD was determined by the method described by Raposo et al. (2008). pH was analyzed using a pH-meter model Crison 20 Basic. C and N were determined through an Elemental Analyser LECO CHNS-932 (Leco Corporation, St Joseph, MI, EEUU). The total neutral sugars were determined colorimetrically according to the anthrone-sulfuric acid assay of Dische (1962).

3. RESULTS AND DISCUSSION

3.1. Determination of kinetic parameters for *Raphidocelis subcapitata* growth

3.1.1. *Kinetic parameters of R. subcapitata growth*

With the aim of determining the kinetic parameters of *R. subcapitata* growth, the experimental data from the production microalgae (X) and substrate consumption coming from batch experiments were adjusted by using the Monod equation (Molazadeh and Danesh, 2019; Rodriguez et al., 2018; Zhao et al., 2016).

Figure 1 shows the evolution of *R. subcapitata* with the cultivation time starting from an inoculum of 13.8 mg chlorophyll/L. As can be observed, the production rate drastically decreased after 7 days, indicating that the exponential period had finished. Therefore, from 1 to 6 days *R. subcapitata* biomass growth could be described according to the exponential equation obtained by the integration of the Monod equation (Rodríguez et al., 2018):

$$X = X_0 \cdot \exp(\mu_{max} \cdot t) \quad (1)$$

Where μ_{max} is the maximum specific growth rate of the microalga (d^{-1}); X is the concentration of the microalga in the medium (mg/L), X_0 is the initial concentration of microalga in the culture medium (mg/L) and t is the cultivation time (d).

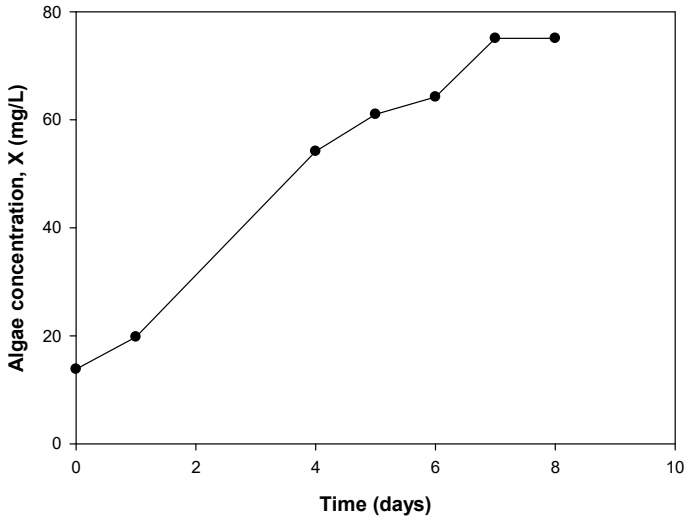


Figure 1. Variation in the concentration of the microalga *Raphidocelis subcapitata* (mg/L) with time (days) in the batch culture of the microalga in the wastewaters coming from the olive oil elaboration process.

In addition, if we assume that substrate limitations are negligible during this period equation (1) can be transformed into:

$$\text{Ln}\,(X/X_0) = \mu_{max} \cdot t \qquad (2)$$

Therefore, the data from the biomass concentration, Ln (X/X_0), and cultivation time could be adjusted for the μ_{max} calculation. Figure 2 shows this linear adjustment. As can be observed, a very good linear correlation was obtained (R^2=0.994; Standard Error of Estimate: 0.098) indicating that the experimental results were adequately described by the Monod model. The line slope led to a value of μ_{max} = 0.31 ± 0.02 $days^{-1}$. On the other hand, the doubling time (the time it takes for the algal population to double the cell

number) (Machado and Soares, 2019) or generation time (g) was calculated using equation (3):

$$g = \text{Ln } 2/\mu_{max} \quad (3)$$

Taking into account the value of μ_{max} and according to Eq. (3), the generation time was 2.23 days.

Although μ_{max} varied with microbial species and light source, this μ_{max}value was somewhat higher than that found for *Spirulina* sp. growth (0.26 $days^{-1}$) in a synthetic medium. In this case, the batch culture experiments were carried out in 50 mL photobioreactors using the F/2 Guillard medium with an excess of

CO_2 in the medium with led lamps (12V/24W) under irradiance of 108 μmol·photons·m^{-2}·s^{-1} (Rodriguez et al., 2018). In addition, a μ_{max} value of 0.41 $days^{-1}$ was found for *Chlorella vulgaris* growth in a medium from effluents of a domestic settling lagoon supplemented with 16% CO_2 and a Nitrogen: Phosporous (N:P) ratio of 10, which used a cool-white fluorescent light illumination and a 12:12 light/dark cycle (Molazadeh et al., 2019).

Similar μ_{max} values to those obtained in the present work were reported by Wang et al. (2016) in batch cultures of *Selenastrum capricornutum* for the simultaneous biogas upgrading and digestate nutrient removal from slurry regardless of the photoperiod. It was demonstrated that for a photoperiod of 16 h light (long), 14 h light (moderate) and 12 h light (short) the maximum specific growth rates were found to be 0.339, 0.341 and 0.326 $days^{-1}$, respectively (Wang et al., 2016).

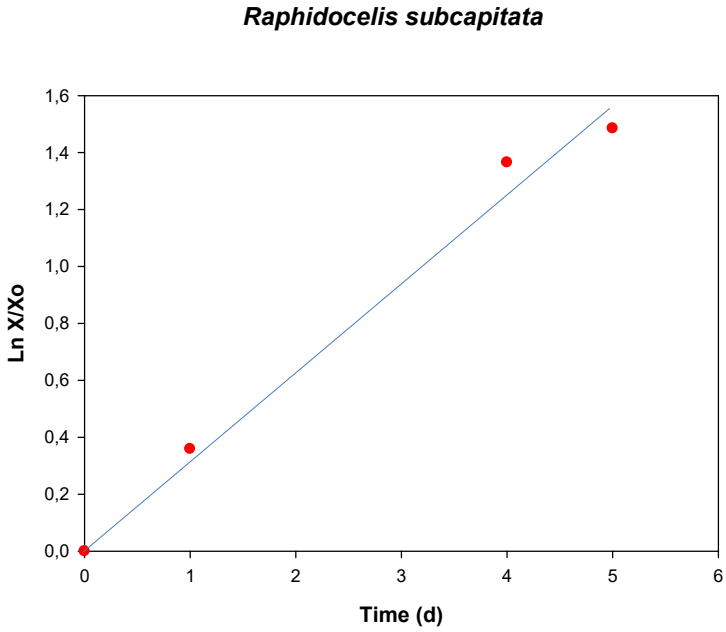


Figure 2. Variation in the Ln (X/X_0) with cultivation time for μ_{max} calculation in the batch culture of *Raphidocelis subcapitata* in the wastewaters coming from the olive oil elaboration process. Where μ_{max} is the maximum specific growth rate of the microalga (d^{-1}); X is the concentration of the microalga in the medium (mg/L), X_0 is the initial concentration of microalga in the culture medium (mg/L)

In the same way, similar and lower μ_{max} values than those obtained in the present work were found in batch cultures of *Selenastrum capricornutum* when different crude oils were present in the medium (Gaur and Kumar, 1981). In this case, *Selenastrum capricornutum* was grown in a modified Hughes medium maintained at 24 ± 1 °C and illuminated by a bank of cool

fluorescent tube lights giving 2400 lux light intensity at the surface of the culture vessels in a 14 h light and 10 h dark cycle (Gaur and Kumar, 1981). The addition of whole crude oils (Assam crude, UAE crude and Bombay high crude) or furnace oil to cultures of *Selenastrum capricornutum* caused a marked reduction in the maximum specific growth rate in a concentration-dependent fashion. More specifically, when the amount of furnace oil added to the culture medium was increased from 2 to 10 µL oil/10 mL culture, the μ_{max} value decreased from 0.37 to 0.13 $days^{-1}$, with the first value being very similar to that obtained in the present work, where this microalga was grown in a mixture of the washing waters of olives and olive oil. Furnace oil was the most toxic to *Selenastrum capricornutum* and was monitored in a decreasing order of toxicity by Assam, UAE and Bombay high crudes. Differences in the chemical compositions of the tested oils were apparently responsible for the variation in toxicity to this and another microalgae such as *Anacystis nidulans*, *Chlorella vulgaris* and *Oocystis sp.*(Gaur and Kumar, 1981).

By contrast, higher mean μ_{max} values of 1.2 $days^{-1}$ were found for *R. subcapitata (Pseudokirchneriella subcapitata*) cultures for nutrient (N and P) removal using water from the North Bosque River (Texas, USA) (Millican et al., 2008). On the other hand, the impact of light quality and quantity on the growth kinetics of *Selenastrum capricornutum* in indoor photobioreactors was

assessed by Gutierrez-Wing et al. (2012). Four commercially available near 400-W artificial light sources for cultures of this microalga were compared in this work: metal halide (MH), high-pressure sodium (HPS), Son Agro and fluorescent. These authors demonstrated that the light elevation and the surface scalar irradiance were shown to have a linear relationship. The lowest μ_{max} value (0.98 $days^{-1}$) observed was obtained with fluorescent light and the highest was obtained with Son Agro (2.39 $days^{-1}$). However, the microalgal culture under Son Agro grew well but crashed prior to 28 days due to wall-growth contamination(Gutierrez-Wing et al., 2012).

3.1.2. Nutrient removal and kinetics for nutrient removal

Figures 3, 4, 5 and 6 show the variation with time in the phosphate, nitrate, total sugars and sCOD concentrations, respectively, in the batch cultures of *R. subcapitata* in the wastewaters used in the present work. As can be seen, the maximum percentages of nutrient removal after 7 days of incubation were found to be 99.7%, 77.6%, 74.1% and 67.8% for phosphate, nitrate, total sugars and sCOD, respectively.

Nutrient removal efficiency is a key indicator for evaluating the efficiency of a microalgal wastewater treatment system (Silva et al., 2015). Nutrient removal by microalgae may be attributed to the

assimilation process of the microalgae, precipitation of the insoluble nutrient, release in the form of gas due to aeration or stirring, and biosorption by algal cells. The microalgal cells take up carbon, nitrogen and phosphorus nutrient elements from the wastewater for the synthesis of nucleic acids, phospholipids and proteins (Katam and Bhattacharyya, 2018). Among the above-mentioned phosphorus removal mechanisms in algal based treatments, the most probable cellular assimilation due to the fact that the pH observed during the operation period was always bellow 7.5, which does not favor precipitation. In this study, phosphate removal (99.7%) was observed (Figure 3) to be higher than that observed for nitrate removal (77.6%). Phosphate removal is mainly associated with nitrogen removal through their respective roles in cellular metabolisms (Katam and Bhattacharyya, 2018). In microalgae, nitrogen is mainly assimilated into proteins which are linked to the production of ribosomes and ribosomal RNA. The uptake of phosphate requires sufficient nitrogen to ensure no restrictions in the protein synthesis of cells.

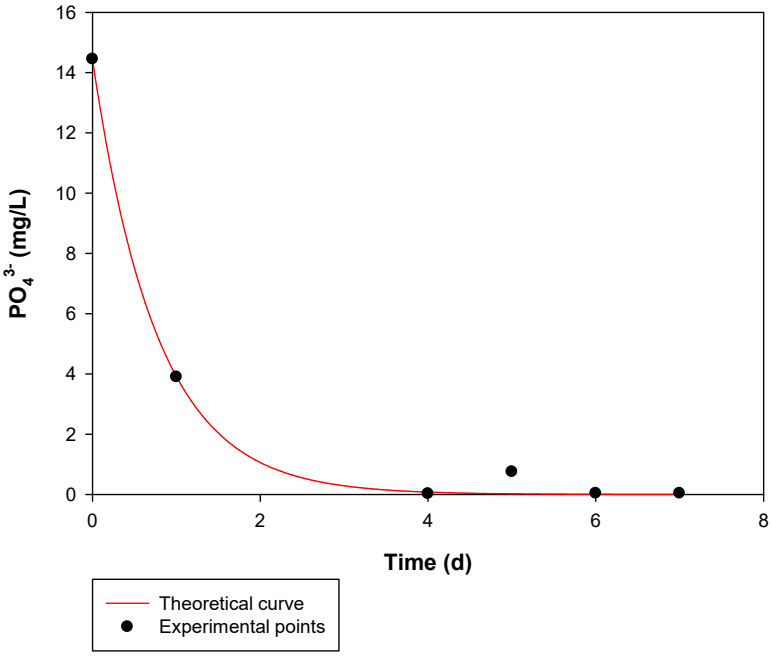


Figure 3. Temporal variation in phosphate concentration (PO_4^{3-}) and theoretical curve, obtained using a pseudo first-order kinetic model, in the batch culture of *Raphidocelis subcapitata* in the wastewaters coming from the olive oil elaboration process.

In general, the phosphorus uptake by microalgae is also affected by many other factors such as algal physiology, phosphate concentration, the chemical form of available phosphate, light intensity and temperature. In the present study, none of the above factors affected phosphate removal. However, lower phosphate removals, 35.78% and 40.95%, were reported by Wang et al.

(2016) in *Selenastrum capricornutum* batch cultures during the simultaneous biogas upgrading and slurry nutrient reduction under high and moderate photoperiods (16 h light : 8 h dark and 14 h light : 10 h dark, respectively). Although it has been reported that in a control medium, *Selenastrum capricornutum* took up phosphate earlier than it grew (Kaneko et al., 2004), it was observed that phosphate removal by this microalga was inhibited by the presence of toxicants such as heavy metals (Pb, Mn, Cr, etc.).

Nitrogen removals in the range of 40-45% were reported by Wang et al. (2016), values also much lower than those obtained in the present work (77.6%)(Figure 4). Nitrogen was mainly removed by assimilating microalgal photosynthesis because microalgal reproduction requires abundant nitrogen to build nucleic acids and proteins (Kumar et al., 2010). Nitrogen assimilation into biomass was the principal mechanism of N removal in the reported study (Wang et al., 2016). De Godos et al. (2010) also reported that green microalgae only removed 21-39% of the total Nitrogen in piggery wastewater.

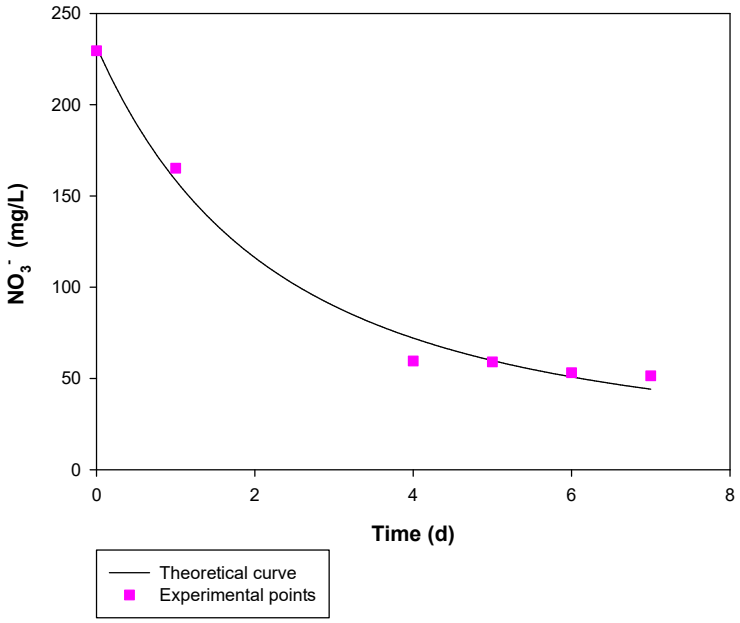


Figure 4. Temporal variation in nitrate concentration (NO_3^-) and theoretical curve, obtained using a pseudo first-order kinetic model, in the batch culture of *Raphidocelis subcapitata* in the wastewaters coming from the olive oil elaboration process.

In relation to organic matter removal, Figures 5 and 6 show that total sugars and soluble COD removals of 74.1% and 67.8% respectively, were found in the present work using *R. subcapitata* and washing waters from olives and olive oil as culture media after 7 days of incubation time. Similar COD removals (70.3%) were reported by Zhao et al. (2016) when *Selenastrum capricornutum*

growth in high-strength synthetic wastewaters was subjected to high nitrogen loading for 14 days of incubation.

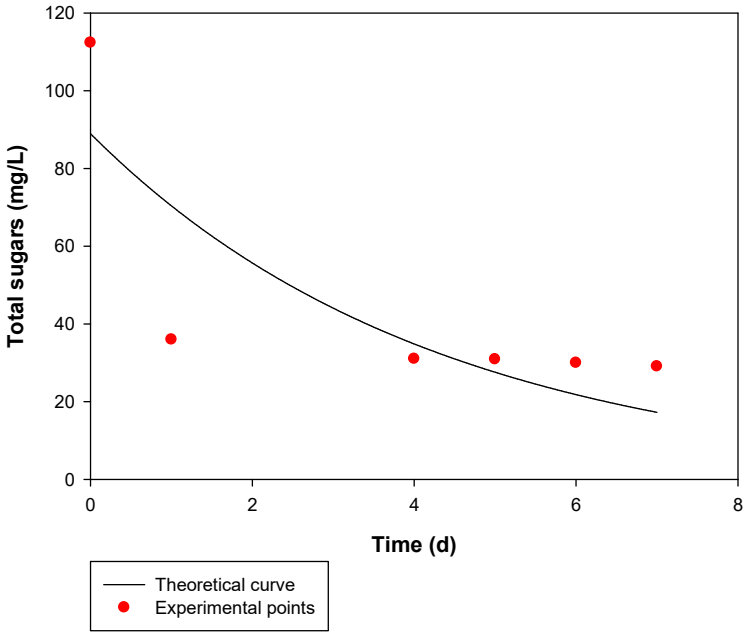


Figure 5. Temporal variation in total sugar concentration and theoretical curve, obtained using a pseudo first-order kinetic model, in the batch culture of *Raphidocelis subcapitata* in the wastewaters coming from the olive oil elaboration process.

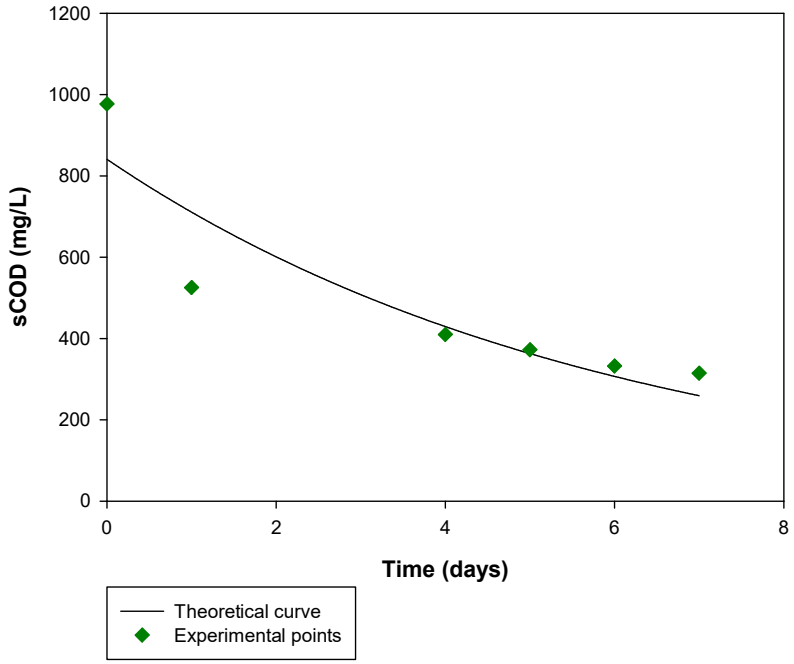


Figure 6. Temporal variation in soluble COD (sCOD) concentration and theoretical curve, obtained using a pseudo first-order kinetic model, in the batch culture of *Raphidocelis subcapitata* in the wastewaters coming from the olive oil elaboration process.

A pseudo-first-order kinetic model was used to describe the temporal variation in nutrient concentration in the cultures (Liu et al., 2017; Silva et al., 2015), which can be described as follows:

$$S = S_0 \cdot e^{-k \cdot t} \quad (4)$$

Where S_0 and S are the nutrient concentrations at the beginning and at time t (days), respectively, and k is the kinetic constant for nutrient removal. Although equation (4) can be linearized to determine the kinetic constant k, this was solved by non-linear regression using the software Sigma Plot (version 11.0). Table 2 shows the kinetic parameters derived from the application of this pseudo-first order kinetic model for phosphate, nitrate, total sugars and sCOD removals. Biokinetic parameters are used in designing biological treatment plants and are determined to understand the rate of nutrient and organic matter utilization (Katam and Bhattacharyya, 2018). Figures 3, 4, 5 and 6 show the experimental data and theoretical curves obtained for the above-mentioned nutrient and organic matter removals. As can be seen in Table 2, the high values for R^2 and the low values for the standard error of estimate for all cases tested demonstrate the goodness of the fit of the experimental data to the model proposed. The kinetic constant for PO_4^{3-} removal was 5 times higher than for nitrate removal and between 5 and 7 times higher than for total sugars and sCOD removals, respectively.

Table 2. Kinetic parameters derived from the application of the pseudo-first-order kinetic model for phosphate, nitrate, total sugars and sCOD removals.

Parameter	S_0 (mg/L)	k (days^{-1})	R^2	S.E.E.*
PO_4^{3-}	14.4 ± 0.3	1.30 ± 0.09	0.9983	0.367
NO_3^-	223 ± 11	0.27 ± 0.02	0.9874	13.439
Total sugars	89 ± 18	0.23 ± 0.09	0.9417	22.146
sCOD	840 ± 98	0.17 ± 0.04	0.9584	97.427

S_0: nutrient concentrations at the beginning; k: kinetic constant; S.E.E.: Standard Error of Estimate

In relation to the phosphate removal, Liu et al. (2017) reported kinetic constant values in the range of 0.93-1.56 $days^{-1}$ using a pseudo first-order kinetic model for the growth of *Chlorella vulgaris* in domestic wastewater with CO_2 (from 1% to 20%) as carbon source. As can be seen, these values were very similar to that found in the present work (1.3 $days^{-1}$), in which *R. subcapitata* grew in washing waters from olives and olive oil derived from the two-phase olive oil manufacturing process.

Regarding nitrate removal, Silva et al (2015) found kinetic constant values in the range of 0.19-0.55 $days^{-1}$ in batch cultures of *Chlorella vulgaris* growth in synthetic media with N:P molar ratios of 16:1 and 24:1. These values were similar to that found in the present work (0.27 $days^{-1}$) with another *Chlorophyta* as *R. subcapitata.*

There are no reports in the literature giving data of kinetic constant values for nutrient removals derived from the mentioned pseudo-first order kinetic model in batch cultures of *R. subcapitata* in either synthetic or real wastewaters.

3.2. Methane yields and study of possible synergic effects

Figure 7 illustrates the variation in the specific cumulative methane production (mL CH_4/g VS) with digestion time for the BMP assays carried out with 100% OMSW, 100% *R. subcapitata*

and for the co-digestion mixtures 75% OMSW-25% *R. subcapitata*, 50% OMSW-50% *R. subcapitata* and 25% OMSW-75% *R. subcapitata*.

The highest methane yield after 33 days of digestion time was 441 mL CH_4/g VS added for the co-digestion of 75% OMSW-25% *R. subcapitata*, while the methane yields obtained for the digestion of the sole substrates were 412 for the AD of the OMSW and 268 mL CH_4/g VS added for the AD of the microalga. Therefore, the methane yield for the above mentioned mixture (75% OMSW-25% *R. subcapitata*) was 7.0% and 64.5% higher than that obtained for the single OMSW and microalga *R. subcapitata,* respectively. Caporgno et al. (2015) found biogas yields of 271 mL biogas/g VS in AD of *Selenastrum capricornutum* at mesophilic temperature. The value obtained for the BMP test of *R. subcapitata* was also of the same order of magnitude as the one found in previous studies on the AD of *Scenedesmus obliquus* (287 mL CH_4/g VS) (Mussgnug et al. 2010). Mussgnug et al. (2010) reported *Scenedesmus* as a microalga with a low degree of decomposition and a high amount of indigestible residues. Although higher biogas productions were reported for freshwater microalgae species, similar results were observed in species characterized by carbohydrate-based cell walls, like in *Selenastrum capricornutum*, *Scenedesmus obliquus* and *Chlorella kessleri* (Caporgno et al., 2015).

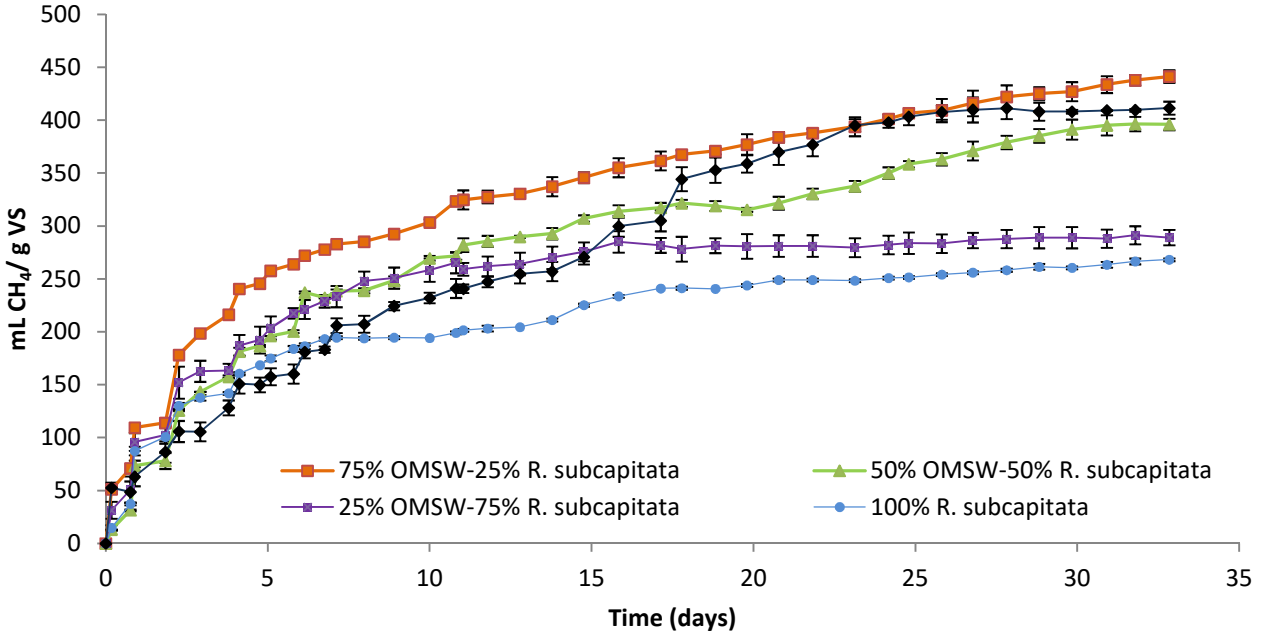


Figure 7. Biochemical methane potential (mL CH_4/g VS added) of 100% olive mill solid waste (OMSW), 100% *Raphidocelis subcapitata (R. subcapitata)* and different co-digestion mixtures.

Nonetheless, the co-digestion of 75% OMSW and 25% *R. subcapitata* showed a high efficiency in methane yield enhancement. By contrast, the methane yields reported by Caporgno et al., (2015) ranged between 330 and 395 mL biogas/g VS for co-digestion mixtures of 75% sewage sludge-25% *Selenastrum capricornutum* and 25% sewage sludge-75% *Selenastrum capricornutum*, respectively. On the other hand, the methane yield recorded by Thorin et al. (2018) in the AD of sole *Selenastrum capricornutum* at mesophilic temperature was 209 ± 5 mL CH_4/g VS, lower than the value achieved in the present work (268 mL CH_4/g VS). However, when *Selenastrum capricornutum* was co-digested with a mixture of waste activated sludge (WAS) and primary sludge (PS) at a ratio of 75% (WAS and PS)-25% *Selenastrum capricornutum,* the methane yield increased up to 303 ± 11 mL CH_4/g VS (Thorin et al., 2018).

The experimental methane yields observed for each co-digestion mixture (Figure 7) were compared to calculate methane yields based on the OMSW, and *R. subcapitata* methane yields separately, according to equation (5).

$$\text{Calculated methane yield (mL } CH_4/\text{g } VS_{added}) = \text{\% OMSW*(412) + \% } R.\ subcapitata \text{ *(268)} \quad (5)$$

Where 412 and 268 are the experimental methane yields (mL CH_4/g VS_{added}) obtained for OMSW and *R. subcapitata*, respectively. % OMSW and % *R. subcapitata* are the percentages of OMSW and *R. subcapitata*, respectively, in each co-digestion mixture. Table 3 summarizes the experimental methane yields obtained for all experiments carried out, as well as the corresponding calculated ones.

Experimental BMP values were higher than the calculated methane yields from equation (5) for some of the co-digestion mixtures tested, showing the occurrence of some synergistic effects (Table 3). For instance, 17.3% for the co-digestion mixture 75% OMSW-25% *R. subcapitata*, and 16.4% for the co-digestion mixture 50% OMSW-50% *R. subcapitata*. Therefore, according to the increase in BMP values, the biodegradability of the above-mentioned co-digestion mixtures was also higher than the biodegradability of the sole substrates.

Table 3. Calculated methane yield values obtained from equation (5), experimental data obtained through biochemical methane potential and improvement in methane yield with respect to its calculated or theoretical value.

OMSW (%)	*Raphidocelis subcapitata (%)*	Calculated (mL CH_4/g VS)	Experimental (mL CH_4/g VS)	Methane yield improvement (%)
100	0	412	412	0
75	25	376	441	17.3
0	50	340	396	16.4
25	75	304	289	0
0	100	268	268	0

OMSW: Olive mill solid waste

3.2.1. Kinetics of methane production

First-order kinetic model

In order to study the process kinetics and estimate the process performance in the AD and co-digestion of the three cases studied, the following first-order kinetic model was used:

$$G = G_m \cdot [1 - \exp(-k \cdot t)] \quad (6)$$

Where G is the cumulative specific methane production (mL CH_4/g VS_{added}), G_m is the ultimate methane production (mL CH_4/g VS_{added}), k is the specific rate constant ($days^{-1}$) and t is the digestion time (days). This kinetic model is normally applied to assess the kinetics of batch AD processes of different types of biodegradable substrates (Li et al., 2012). This model is based on the assumption that methane production is proportional to the amount of substrate and not limited by microbial cell mass (Wang et al., 2017).

Table 4 summarizes the kinetic parameters obtained from Eq. (6) for the different co-digestion mixtures tested and for the two substrates studied individually. Values situated after ± represent the standard deviation of each parameter. Error was defined as the percentage difference between the experimental and the predicted or theoretical methane yield coefficient. As can be seen, errors or

deviations between the experimental G_m values (Figure 1) and the theoretical ones (Table 4) lower than 12.4% were obtained for all the cases studied. In addition, the low values for the standard deviations and the high determination coefficient values prove the appropriate fit of the experimental results to the proposed model. As an example, Figure 8 shows the experimental data of methane production and digestion time for the mixture 25% OMSW-75% *R. subcapitata*, and the theoretical curve of the adjustment to this first order kinetic model.

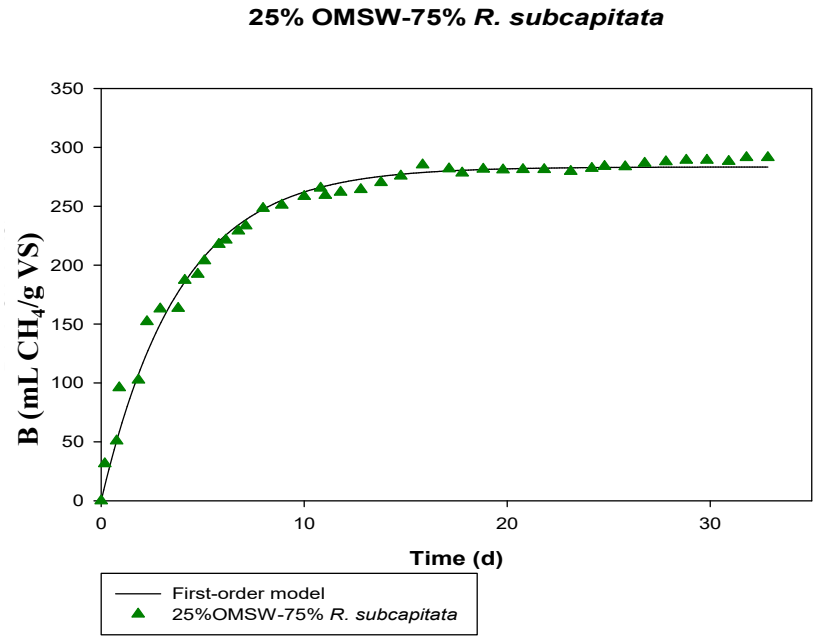


Figure 8. Variation in the experimental methane production with time for the mixture 25% Olive mill solid waste (OMSW)-75% *Raphidocelis subcapitata* and theoretical curve obtained from the first-order kinetic model.

As can be seen in Table 4, the ultimate methane production did not increase with respect to the value found for the sole OMSW when co-digested with *R. subcapitata* for the different percentages of mixtures tested.

The kinetic constant, *k*, increased by 271% and 18% when the OMSW is co-digested with *R. subcapitata* at a ratio of 25% OMSW-75% *R. subcapitata* compared with the values achieved for single OMSW and sole *R. subcapitata*, respectively. By contrast, the co-digestion of *Salvinia molesta* (SM), one of the free-floating aquatic weeds (which can grow very rapidly) with rice straw (RS) at different mixture ratios (from 40:60 to 0:100, SM:RS) at different initial pH values (6-8) gave the same first-order kinetic constant values (0.01 $days^{-1}$) regardless of the concentration of SM and RS in the mixture and initial pH (Syaichurrozi et al., 2018). In all cases, these kinetic constant values were much lower than those obtained in the present work (Table 4).

Table 4. Values of the first-order kinetic constant, ultimate methane yields of the anaerobic digestion of the single olive mill solid waste (OMSW), sole *Raphidocelis subcapitata*, and different co-digestion mixtures tested.

Substrate	G_{max} (mL CH_4/g VS)	k ($days^{-1}$)	R^2	S.E.	% Error
100% OMSW	461 ± 13	0.07±0.00	0.9882	19.42	12.4%
75% OMSW-25% *R. s.*	404 ± 7	0.17±0.01	0.9776	24.50	7.5%
50% OMSW-50% *R. s.*	372 ± 6	0.13±0.00	0.9863	18.33	6.0%
25% OMSW-75% *R. s.*	283 ± 2	0.26±0.01	0.9925	9.47	2.7%
100% *R. s.*	274 ± 4	0.22±0.01	0.9819	16.22	7.8%

R. s.: Raphidocelis subcapitata; OMSW: olive mill solid waste; G_{max}: experimental values; k: kinetic constant; R^2: coefficient of determination; S.E.: Standard error of estimate; %Error: difference between measured and predicted methane yield values.

Transference Function model

The Transference Function (TF) model was also applied to fit the experimental data of methane production during BMP tests (eq. 7). The transference function *(Reaction curve-type model*), used mainly for control purposes, considers that any process might be analyzed as a system receiving inputs and generating outputs (Donoso-Bravo et al. 2010). The TF model was successfully applied by several authors for the biomethanization of different organic wastes (Donoso-Bravo et al. 2010; Li et al. 2012;). The TF model is given by the following expression:

$$B = B_{max} * \left(1 - exp\left[-\frac{R_{max}(t-\gamma)}{B_{max}}\right]\right) \quad (7)$$

Where B (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, R_{max} is the maximum methane production rate (mL CH_4/(g VS_{added}·d)), t (d) is the digestion time and γ (d) is the lag time.

Error (%), determination coefficient (R^2) and standard error of estimate were calculated to evaluate the goodness-of-fit and the accuracy of the results. Error was defined as the percentage

difference between the experimental and the predicted or theoretical methane yield coefficient.

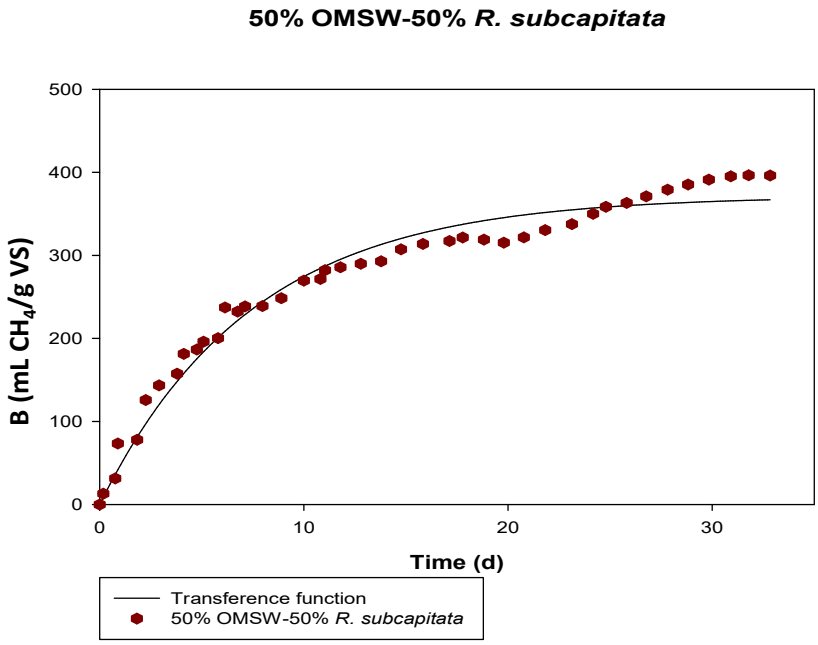


Figure 9. Variation in the experimental methane production with time for the mixture 50% Olive mill solid waste (OMSW)-50% *Raphidocelis subcapitata* and theoretical curve obtained from the Transference function model.

The kinetic parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 11) (Table 5). As can be seen in Table 5, the low

values of the standard deviations and the high determination coefficient values demonstrate the appropriate fit of the experimental results to the proposed model. As an example, Figure 9 shows the experimental data of methane production –digestion time for the mixture 50% OMSW-50% *R. subcapitata*, and the theoretical curve of the adjustment of these points to the TF model.

Among the different co-digestion mixtures assayed, the highest maximum methane production rate, Rm, was obtained from the mixture 25% OMSW-75% R. subcapitata with a value of 73.3 ± 2.3 mL CH4/(g VSadded·d). This value was 3.9 and 46.8% higher than those obtained for 75% OMSW-25% R. subcapitata and 50% OMSW-50% R. subcapitata, respectively. In addition, it was 114.9 and 32.7% higher than those achieved through single OMSW and sole R. subcapitata.

Table 5: Values for the parameters obtained from the Transference Function model for the different substrates studied.

Substrate	B_{max} **(mL CH_4/g VS)**	R_{max} **(mL CH_4/g VS·d)**	**ʎ**	**R^2**	**S.E.E.**	**% Error**
100% OMSW	460 ± 15	34.1 ± 1.9	$1.0 \cdot 10^{-8}$	0.9882	19.67	11.0%
75% OMSW-25% *R. s.*	404 ± 7	70.5 ± 4.6	$6.6 \cdot 10^{-9}$	0.9766	24.81	7.5%
50% OMSW-50% *R. s.*	371 ± 6	49.9 ± 2.7	$4.3 \cdot 10^{-9}$	0.9863	18.57	6.3%
25% OMSW-75% *R. s.*	283 ± 2	73.3 ± 2.3	$1.6 \cdot 10^{-11}$	0.9925	9.60	2.7%
100% *R. s.*	247 ± 4	55.2 ± 4.0	$4.3 \cdot 10^{-9}$	0.9719	16.43	7.8%

OMSW: olive mill solid waste; R. s.: Raphidocelis subcapitata; Bmax: ultimate methane production; *Rmax*: maximum methane production rate, ʎ:calculated lag times; S.E.E.: Standard Error of Estimate; %Error ((Bmax experimental – Bmax model)/Bmax experimental)·100

On the other hand, a decrease in the maximum methane production rate, R_m, from 73.3 to 55.2 mL CH_4/(g VS_{added} d) was observed when the percentage of *R. subcapitata* increased from 75% to 100%. In the same way, it was recently reported that the values for R_m obtained by the co-digestion of *Chlorella vulgaris* with potato processing waste were gradually decreased as the proportions of *Chlorella* augmented in the mixture from 25% to 75% (on VS basis) (Zhang et al. 2019).

These results again reflect that the co-digestion of microalgae with carbon-rich co-substrates (i.e. food wastes) had a relatively high impact on microalgal anaerobic biodegradability and conversion rate (Zhen et al. 2016).

Conclusions

This study indicates that *Raphidocelis subcapitata* (*R. subcapitata*) is capable of growing in washing waters from olives and olive oil from the two-phase olive oil manufacturing process and has the potential to remove organic carbon and nutrients (99.7% phosphate and 77.6% nitrate). The maximum specific growth rate of this microalga in the mentioned washing wastewater was found to be 0.31 ± 0.02 $days^{-1}$. A pseudo-first-order kinetic model allowed for describing the temporal variation in nutrient concentrations in the culture. Specifically, the rate of phosphate

removal (1.30 ± 0.09 $days^{-1}$) was around six times higher than that obtained for nitrate (0.27 ± 0.02 $days^{-1}$), total sugars (0.23 ± 0.09 $days^{-1}$) and soluble chemical oxygen demand (0.17 ± 0.04 $days^{-1}$).

Anaerobic co-digestion of the mixture 75% OMSW – 25% *R. subcapitata* increased the methane yield 7.0 and 64.5% compared to the anaerobic digestion of 100% OMSW and 100% *R. subcapitata,* respectively, and to the other mixture percentages tested, closing the loop of the two-phase olive oil production system for resource efficiency and environmental management. In addition, this co-digestion mixture had a higher synergic effect than the other co-digestion mixtures studied. However, the mixture 25% OMSW-75% *R. subcapitata* showed the highest specific rate constant and maximum methane production rate compared to the other mixture percentages tested.

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Chapter 8

Reuse of the digestate derived from biomethanization of two-phase olive mill solid waste as soil amendment or fertilizer for cultivation of forage grass (*Lolium rigidum* var. *Wimmera*).

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M.J. Fernández-Rodríguez; J.M. Mancilla-Leytón; A. Jiménez-Rodríguez; B. Rincón; R. Borja, Reuse of the digestate derived from biomethanization of two-phase olive mill solid waste as soil amendment or fertilizer for cultivation of forage grass (*Lolium rigidum* var. *Wimmera*).

Abstract

Olive mill solid waste (OMSW) is the main by-product from the olive oil production process. It is a very polluting by-product, not only because of its characteristics, but also because of the large amount of OMSW which is generated each year, 2 to 4 million tonnes/year in Spain. The anaerobic digestion of this by-product is a well-studied process, resulting in the generation of biogas, a mixture of methane and carbon dioxide mainly of high calorific values (20-25 MJ/m^3), and a effluent or digestate. However, the digestate of this by-product has never been characterized. This study provides information on how the composition of OMSW digestate shows promising implications as a soil amendment or fertilizer due to the quality of the biomass from *Lolium rigidum*, a useful grass specie for the production of forage. Three OMSW digestate alternative ways of application or treatments were investigated, the digestate and the solid fraction of the digestate for a nutrient-poor soil amendment and the liquid fraction of the digestate as fertilizer. The results confirm that all OMSW digestate treatments studied presented suitable characteristics for agricultural use, showing an optimal Carbon/Nitrogen ratio, adequate values for heavy metals below the limits established by the Spanish and European legislation and absence of pathogens. However, fertirrigation was the treatment that provided *Lolium rigidum* with

the best characteristics, improving its shoot biomass, photosynthetic rate and nutritional content.

1. Introduction

The great health benefits of olive oil have led to a worldwide tendency toward its consumption (Gavahian et al., 2019). In Spain alone the olive oil production was of 1,047,100 tonnes at the end of the 2017/18 season (MAPA, 2019). Such increases in olive oil consumption have brought, and continue to bring changes in the sector (Neves and Pires, 2019). In the 1990s, the production of olive oil underwent an improvement by introducing the two-phase olive oil system. This new system entailed a reduction in the use of water as well as a reduction in energy consumption during the process and a marked improvement in the quality of the product (Alburquerque et al., 2004). The modernization of the sector brought a reduction in the volume of wastewaters generated but, by contrast, large amounts of olive mill solid waste (OMSW) are produced. It is estimated that in Spain alone, between 2 and 4 tons of OMSW are generated each year in a short period of time (November to September) (Borja et al., 2006). The OMSW is characterized as having a high organic matter content, a moisture content of around 70%, as well as an acidic pH as a consequence of its phenol, polyalcohol and sugar contents (Borja et al., 2006). The large amount of OMSW generated each season and its characteristics represent an environmental hazard, so the

valorization of this by-product is certainly necessary in order to reduce the impact that olive oil industries have on the environment (Borja et al., 2006). The valorization of this type of by-product is an increasing trend in order to comply with the guidelines established by the European Comission in 2015 where the main axes are reduce, reuse and recycle (European Comission, 2015).

Anaerobic digestion (AD) is a key tool for organic waste treatment, since organic matter is mineralized, in absence of oxygen, by the action of a consotium of microorganisms and, therefore, the pollution load of this type of waste is reduced (Abad et al., 2019). After the AD process, two main a biogas (mixture of CH_4 and CO_2, mainly) with a high concentration of methane and high calorific value is produced during the AD process. In addition to biogas, a semi-stabilized by-product with a high moisture content (digestate) is generated (O'Brien et al., 2019). During the AD process, both nitrogen and phosphorus are mineralized, but they are not eliminated from the system, so the digestate is characterized as being rich in nitrogen and phosphorus, hence its great potential for use as soil amendment or fertilizer (Fernández-Bayo et al., 2017).

Verdi et al. (2019) define the use of digestate as fertilizer as a great opportunity to reduce the environmental impact derived from mineral fertilization; the fertilization from digestate could be complementary, and in some cases, it is possible to obtain synthesis

fertilizers and optimize the profitability of farms in the medium term. The quality and characteristics of this digestate depend on the substrate used in AD (Solé-Bundó et al., 2017). Currently, most of the studies carried out with digestates from the AD are of cattle manure o pig slurry (Bustamante et al., 2019, Montemayor et al., 2019, O'Brien et al., 2019,). These digestates have high nitrogen concentrations and the added handicap that they require a sterilization process in order to eliminate pathogens (Qi et al., 2019). The AD of OMSW is a well-studied process, from which values of up to 321 ml CH_4/g VS_{added} are obtained (Fernández-Rodríguez et al., 2019). The use of digestate would bring another added value to the OMSW AD process and provide sustainable waste management. At present, the use of digestates has not been exploited efficiently and it is necessary to continue investigating the possible ways to recycle this by-product. In addition, the use of OMSW digestate has not yet been investigated.

The aim of this study was to evaluate the influence of the OMSW digestate, for the first time, as soil amendment and fertilizer and its influence on the growth of a Mediterranean herbaceous specie, *Lolium rigidum*, common in pastures with high forage value and frequently used as feed for cattle.

2. Materials and methods

2.1. Digestate

Digestate is the semi-liquid by-product resulting after the AD. The digestates used were collected after the AD of olive mill solid waste (OMSW) carried out at mesophilic temperature in batch mode. Anaerobic reactors were inoculated with biomass obtained from an industrial up-flow anaerobic sludge blanket reactor from a brewery located in Sevilla (Spain). The inoculum to substrate ratio in the AD was 2 (Volatiles Solid basis).

The OMSW was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). Olive stone pieces were removed using a 2 mm mesh before AD process.

2.2. Digestate characterization

Total solids (TS), Volatile solids (VS), soluble chemical oxygen demand (SCOD), total alkalinity (TA) and total Kjeldahl nitrogen (TKN) were analyzed in accordance with Standard methods (American Public Health et al., 2005). Chemical oxygen demand (COD) was determined by the method described by Raposo et al. (2008). pH was analyzed using a pH-meter model Crison 20 Basic. C and N were determined through an Elemental Analyzer LECO

CHNS-932 (Leco Corporation, St Joseph, MI, EEUU). Heavy metals were analyzed by the method

2.3. Specie selection

A Mediterranean herbaceous species, *Lolium rigidum* var. *Wimmera*, common in pastures with high forage value and frequently used as feed for cattle was selected for the experiments (Leiva et al., 1997). It is an annual specie, well adapted to the dry conditions of the Mediterranean area and with a high palatability and high nutritional value.

2.4. Experimental design

In order to determine the quality of the OMSW digestate for agricultural reuse as a fertilizer, and its influence on the growth and development of seedlings, different strategies or treatments were established:

(i) Direct use of the raw OMSW digestate for amendment of a nutrient-poor soil. Different mixtures of the OMSW digestate (D) and silica inert substrate were tested: D1/3: 25% D-75 % silica and D1/4: 20% D-80% silica,

respectively and irrigated with Hoagland nutrient solution at 5% (Hoagland and Arnon, 1938).

(ii) Use of the solid fraction (SD) of the OMSW digestate obtained after centrifugation at 2000 rpm, 2 min for amendment of a nutrient-poor soil. The SD obtained after centrifugation was used in a mixture 25% SD- 75% silica inert substrate and irrigated with Hoagland nutrient solution at 5%.

(iii) Use of the liquid fraction of the OMSW digestate obtained after centrifugation at 2000 rpm, 2 min as fertilizer of a nutrient-poor soil. The fertirrigation tests were carried out in two set of pots with silica inert substrate and irrigated with Hoagland nutrient solution at 5%. The first set of pots was fertirrigated once (F1) with the liquid part of the OMSW digestate after 15 days of the beginning of the experiment. The second set of pots with silica inert substrate and irrigated with Hoagland nutrient solution at 5%, was fertirrigated twice (F2) with the liquid part of the OMSW digestate after 15 and 30 days of the beginning of the experiments.

Two sets of control pots with silica inert substrate and irrigated with Hoagland nutrient solution at 5% (Control H5%) and 20% (control H20%) were used. The main objective of using these two controls was to compare the *Lolium rigidum* growth in a nutrient-poor soil (silica and H5%) with a nutrient-rich soil (silica and H20%).

All OMSW digestate mixtures used in the experiment were in accordance with the limits established by the European Nitrates Directive (EEC, 1991)

Each set of treatments contained 5 replicate pots (0.6 L). In each pot, ten seeds were placed and covered with substrate (1–2 cm). The pots were placed in a glasshouse with minimum-maximum temperatures of 24–26 °C, 40–60% relative humidity and natural daylight (minimum and maximum light flux: 200 and 1000 μmol m^{-2} s^{-1}, respectively). The pots were checked over a period of 50 days for emergence of photosynthetic tissues above the substrate level. We considered that the seedling had emerged once the stem had reached 1 cm in height.

Three parameters of emergence were determined: final emergence percentage, time to first emergence and mean time to emergence (MTE), calculated as: $MTE = \Sigma_i (n_i \times d_i)/N$, where *n* is the number of seeds emerged at day *i; d* the incubation period in days; and *N* is the total number of seeds emerged in the treatment (Mancilla-Leytón et al., 2012a)

After 50 days the sowing and gas exchange were determined. Likewise, seedling height (stem base to maximum height of horizontal leaves), dry root and shoot biomass were measured in each treatment.

2.5. Gas exchange

Gas exchange measurements were taken from randomly selected, fully expanded leaves (n = 12, two measurements per pot plus two additional random measurements), using an infrared gas analyzer in an open system (Li-6400-XT, Li-COR Inc., Neb., USA). Net photosynthetic rate (A), intercellular CO_2 concentration (Ci) and stomatal conductance to CO_2 (Gs) were determined at an ambient CO_2 concentration of 400 ppm CO_2, temperature of 20/25 °C, 50 ± 5% relative humidity and a photon flux density of 1000 μmol m^{-2} s^{-1}. Values for the parameters A, Ci and Gs were calculated using the standard formulae of von Caemmerer and Farquhar (1981).

2.6. Mineral analysis

At the end of the experimental period, leaf samples were dried at 80 °C for 48 h and ground. Then, 0.25 g of sample were digested with 4 mL HNO_3, 0.5 mL HF and 1 mL H_2O_2, shaking well to wet the sample completely. Measurements of Mg, K, Ca, S and P were taken by inductively coupled plasma (ICP-OES) (VARIAN ICP 720-ES). Total N-Kjeldahl, samples were treated with concentrated sulfuric acid in the presence of catalyst (mixture of Se and K_2SO) at 380 °C for 2 h. Ammoniacal nitrogen was determined by the

indophenol method in a Bran + Luebbe AIII autoanalyzer. The results were expressed as % $N\text{-}NH_4^+$ of dry matter.

2.7. Pathogens analyses

According to Spanish Law (RD 1620/2007) for water reuse as irrigation of pastures for animal consumption, nematodes, *Taenia saginata, Taenia solium*, *Escherichia coli* (*E. coli*) and presence / absence of pathogens such as *Salmonella* were determined in the OMSW digestate. *E. coli* and *Salmonella* were determined according to UNE-EN ISO 9308-1:2014, nematodes and taenia were determined using the isolation method by classical extraction in Whitehead tray and inverted microscopy.

2.8. Statistics

Normality and homogeneity of variances were tested using the Kolmogorov-Smirnov and Levene tests, respectively, in order to assume the hypotheses necessary to perform parametric tests. One way ANOVA was used to examine the significant differences of each variable. Tukey's (post hoc) test was performed for multiple comparisons. The non-parametric equivalent, the Kruskal-Wallis test, was employed when was necessary. IBM 25.0 for Windows

(SPSS Statistic, Inc., Chicago, IL, USA) was used for all the statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Digestate characterization

3.1.1. Main chemical parameters

The main characteristics of the OMSW digestate are shown in Table 1. The pH of the OMSW digestate was 7.91 ± 0.16, similar to that reported by O'brien et al. (2019) for the digestate of dairy manure and food waste anaerobic co-digestion (pH = 8.4) and more alkaline than the pH reported by Solé-Bundó et al. (2017) for the digestate of microalgae and primary sludge anaerobic co-digestion (pH = 7.30 ± 0.15). This value is within the normal pH range of the soil of the Mediterranean area (Verheye and de la Rosa, 2005). The TS content of the digestate was 2.80 ± 0.08% with 73.8% of VS. The carbon/nitrogen (C/N) ratio of the OMSW digestate (C/N = 11 ± 0.8) was higher than the C/N ratio of the microalgal digestate (C/N = 3.17) (Solé-Bundó et al., 2017), but similar to that reported by O'brien et al. (2019) for the anaerobic co-digestion digestate (C N = 15.3). The characteristics of digestate depend on the substrate used during the AD process (Solé-Bundo et al., 2017). The C/N contents of microalgae are between 5 and 12 (Klassen et al., 2015).

On the other hand, one of the benefits of anaerobic co-digestion is that by using two or more substrates, the C/N ratios are balanced. The OMSW C/N ratio is 31.4 (Fernández-Rodríguez et al., 2014), near of the range established as optimum for the AD process (C/N = 20-30) (Xu et al., 2017). The OMSW digestate C/N ratio was 11 ± 0.8, which implies a lower nitrogen content compared to the microalgal digestates (C/N = 3.17) (Solé-Bundo et al., 2017) or cattle manure digestates (C/N = 1.2) (Alburquerque et al., 2012). The C/N ratio of the OMSW digestate was within the range described by Fernández-Bayo et al. (2017), as a typical value for stable organic materials. The total nitrogen content of the OMSW digestate was 1.14 ± 0.12 g L^{-1} (Table 1), similar value to the one assigned to co-digestion digestates of thermally pretreated microalgae with primary sludge (Sólé-Bundó et al., 2017).

Table 1. Characteristics of the digestate olive mill solid waste (OMSW) used in the experiment. TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, SCOD: soluble chemical oxygen demand, TKN: total Kjeldahl nitrogen, and C/N: carbon/nitrogen.

Parameter	**OMSW digestate**
TS (%)	2.8 ± 0.1
VS (%)	2.1 ± 0.0
VS/TS (%)	74 ± 2
COD (g O_2/L)	27.76 ± 1.48
SCOD (g O_2/L)	3.65 ±0 71
pH	7.91 ± 0.2
C/N ratio	11.0 ± 0.8
TKN (g L^{-1})	1.14 ± 0.12

3.1.2. Heavy metals and pathogens

In order to evaluate the risk of soil contamination and the contamination of *Lolium rigidum* var. *Wimmera* for use in animal feed, the heavy metal concentration and the presence of pathogens were analyzed in the OMSW digestate. Animal feeding can subsequently influence human health, since animals eating contaminated pastures can cause diseases in humans (Hinton, 2010). The main infectious health hazards associated with feed and forage which pose a hazard to consumers of foods of animal origen are the high concentrations of heavy metals and the presence of microorganisms such as *Salmonella*, *Escherichia coli*, etc. (Hinton, 2010). The heavy metal concentrations detected in the OMSW digestate (Table 2) were below the limits established as toxic by Spanish law (RD1310/1990), and also by the EU Directive (CEC 2003). Besides this, no pathogens were detected in the OMSW digestate, nematodes: 0 egg L^{-1}, *Taenia saginata,* and *Taenia solium:* 0 egg/L^{-1}, *E. coli*: 0 colony forming units/L and no presence of pathogens such as *Salmonella* were determined in the OMSW digestate.

3.2. Effect of OMSW digestate on *Lolium rigidum* var. *Wimmera* emergence

The effect of different treatments on *Lolium rigidum* emergence was assessed and was compared with the emergence of this Mediterranean herbaceous in the control experiments (H5% and H20%). The emergence rate of D1/3 was 82 ± 4%, for the treatment D1/4 it was 78 ± 6%, 10 ± 6% for the solid fraction of the digestate (SD) and 83 ± 4% and 84 ± 4% for the F1 and F2 treatments, respectively. No statistically significant differences ($p > 0.05$) were found between the emergence rates of the different treatments (79-84%), except for the treatment SD (10%) (Table 3). In all treatments the emergence time for *Lolium rigidum* was 5 days, except for treatment SD, where the first emergence was delayed until day 23 (Table 3). As can be seen, there was no phytotoxic effect of the OMSW digestate on the emergence of *Lolium rigidum*, since no significant differences were found among the treatments (F1, F2, D1/3 and D1/4) and the controls (H5% y H20%). The only treatment that negatively affected the emergence of *Lolium rigidum* was the solid fraction of the OMSW digestate (SD) (Table 3). SD treatment required significantly more time to produce photosynthetic tissue due to the digestate solid fraction content being slower to release organic matter or the lignocellulosic biomass being resistant to microbial degradation (Möller and Müller, 2012), so a clear delay in *Lolium rigidum* emergence was

observed (Table 3). On the other hand, the other treatments did not show a negative effect on the emergence of *Lolium rigidum*. In previous studies, environmental factors such as pH, soil salinity and temperature were identified as the main factors that affect the emergence of rigid ryegrass (*Lolium rigidum*) (Rahman and Asaduzzaman, 2019). In addition, Solvåg Nesse et al. (2018) showed that nitrogen is one of the main causes of emergence inhibition in seeds. Values above 3% of total nitrogen and a pH between 7.5 and 8 have been described as inhibiters for seed emergence (Solvåg Nesse et al., 2018). In this work, the total nitrogen content of the digestate was below 3%, although the pH was 7.91, no *Lolium rigidum* seed emergence inhibition was observed.

Table 2 Concentration of heavy metals in the shoots and roots of *Lolium rigidum* var. *Wimmera* grown in the different OMSW digestate treatments. Where D: raw digestate for amendment of a nutrient-poor soil, D1/3: mixture 25% D-75 % silica and D1/4: mixture 20% D-80% silica, respectively. H5% and H20% are Hoagland nutrient solution at 5% and 20% concentration, respectively, and F1 and F2 are the liquid fraction of digestate used as fertilizer with one and two fertirrigates, respectively. OMSW: olive mill solid waste.

OMSW digestate tretamets	***Lolium rigidum* part**	**Cd** $mgKg^{-1}$	**S** $mgKg^{-1}$	**Co** $mgKg^{-1}$	**Ba** $mgKg^{-1}$	**Cu** $mgKg^{-1}$	**Pb** $mgKg^{-1}$	**Mo** $mgKg^{-1}$
D1/3	shoot	<0.10	0.26	0.95	13.96	10.53	<0.10	11.12
D1/4	shoot	<0.10	0.22	1.14	11.35	11.17	<0.10	11.74
F1	shoot	<0.10	0.29	3.04	14.55	8.41	<0.10	8.96
F2	shoot	0.01	0.45	3.67	8.15	6.41	0.24	13.40
H20%	shoot	0.06	0.32	1.02	8.98	7.03	<0.10	1.79
H5%	shoot	0.02	0.21	0.80	18.13	11.53	0.60	2.32
D1/3	root	0.06	0.20	7.39	11.68	27.08	1.40	6.01
D1/4	root	0.08	0.14	6.27	15.04	12.85	1.74	5.23
F1	root	0.07	0.15	16.52	61.49	12.09	3.23	5.53
F2	root	0.03	0.40	21.33	155.23	13.74	2.62	10.23
H20%	root	<0.10	0.16	3.46	9.25	9.73	0.37	0.85
H5%	root	<0.10	0.13	2.75	9.01	13.75	2.35	0.78
Limit values*		10	10	40	40	40	40	40

OMSW digestate tretamets	***Lolium rigidum* part**	**V**	**As**	**Ni**	**Al**	**B**	**Cr**	**Mn**	**Zn**
		$mgKg^{-1}$	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**	**$mgKg^{-1}$**
D1/3	shoot	0.81	<0.10	8.00	32.52	41.06	16.32	179.08	57.89
D1/4	shoot	1.45	<0.10	12.68	37.39	45.49	29.09	336.93	63.91
F1	shoot	0.55	<0.10	9.63	31.85	50.95	15.40	177.14	40.92
F2	shoot	1.59	<0.10	15.42	56.88	107.32	25.20	105.87	39.51
H20%	shoot	0.36	<0.10	14.95	41.02	22.06	30.62	66.46	27.99
H5%	shoot	1.00	<0.10	9.44	29.51	11.22	16.96	109.66	55.64
D1/3	root	7.87	<0.10	121.51	360.76	9.55	353.09	174.12	39.06
D1/4	root	7.13	<0.10	66.45	501.67	5.49	165.84	142.16	39.45
F1	root	9.78	<0.10	62.58	315.57	15.86	141.39	345.20	32.17
F2	root	16.17	<0.10	63.20	341.21	53.69	137.27	540.48	48.08
H20%	root	2.78	<0.10	50.46	459.41	3.72	127.63	66.96	21.18
H5%	root	2.01	<0.10	34.39	342.61	1.14	89.49	69.89	38.26
Limit values*		40	400	400	1000	1000	1000	1000	1000

*Limit values according to AFFCO, 1996

Table 3. Percentage of total emergence of *Lolium germination* (%), time to first emergence (days) and mean time to emergence (MTE, days) for each treatment. Where D: raw digestate for amendment of a nutrient-poor soil, D1/3: mixture 25% D-75 % silica and D1/4: mixture 20% D-80% silica, respectively. H5% and H20% are Hoagland nutrient solution at 5% and 20% concentration, respectively, and F1 and F2 are the liquid fraction of digestate used as fertilizer with one and two fertirrigates, respectively.). In each column, different letters indicate means that are significantly different from each other (Tukey test, $p < 0.05$).

	%	**First emergence (days)**	**MTE (days)**
H5%	82 ± 2 a	5 ± 0 a	8 ± 1
H20%	80 ± 3 a	5 ± 0 a	8 ± 1
F1	83 ± 4 a	5 ± 0 a	7 ± 0
F2	84 ± 4 a	5 ± 0 a	7 ± 1
D1/3	82 ± 4 a	5 ± 0 a	8 ± 2
D1/4	78 ± 6 a	5 ± 0 a	8 ± 1
SD	10 ± 6 c	23 ± 4 b	23 ± 4

3.3. Effect of OMSW digestate on biometric characteristics

The effect of the different treatments on the total biomass, on the shoot biomass and on the root biomass was assessed. The effect of the different treatments on the total biomass of *Lolium rigidum* was evaluated (Figure 1A). D1/3 and D1/4 showed similar values, D1/3= 0.609 ± 0.017 and D1/4= 0.625 ± 0.029g dry matter (DM) to that obtained for the control H20% (0.690 ± 0.020g DM) and were significantly higher than those obtained for all other treatments (H5% = 0.187 ± 0.005 g DM, F1 = 0.324 ± 0.019 g DM, F2 = 0.502 ± 0.015 g DM)($F=106.86$, $p < 0.001$). The H5% control showed the lowest total biomass production, with once again no phytotoxicity observed with the use of the OMSW digestate (Figure 1A).

Shoot biomass was also affected by the different treatments used with significant differences among them ($F=50.16$ $p < 0.001$). The seedlings grown in the pots treated with F2 presented shoot biomass values similar to those found for the H20% control seedlings and the maximum values reached during the experiment (H20%= 0.298 ± 0.011 and F2= 0.282 ± 0.005g DM); the H5% control was the one with the lowest shoot biomass (0.077± 0.004 g DM) (Figure 1D).

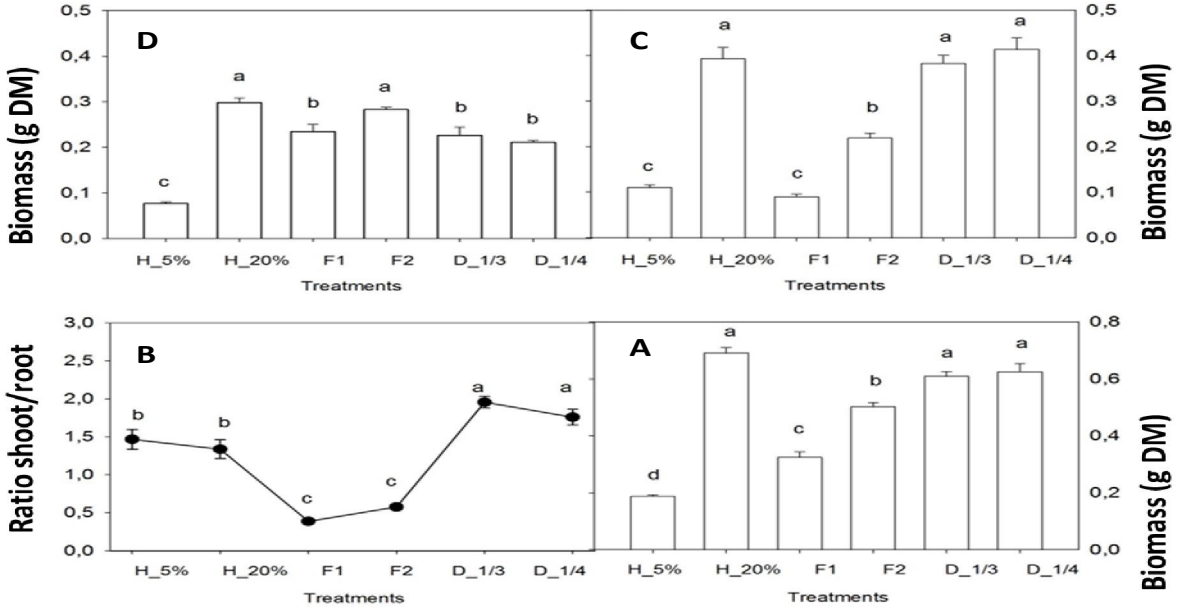

Figure 1 Total biomass (A), root/shoot ratio (B), shoot biomass (C) and root biomass (D) of *Lolium rigidum* var. *Wimmera* grown in the different OMSW digestate treatments. Where D: raw digestate for amendment of a nutrient-poor soil, D1/3: mixture 25% D-75 % silica and D1/4: mixture 20% D-80% silica, respectively. D: RawH 5% and H20% are Hoagland nutrient solution at 5% and 20% concentration, respectively, and F1 and F2 are the liquid fraction of digestate used as fertilizer with one and two fertirrigates, respectively. Different letters indicate means that are significantly different from each other (Tukey, $p < 0.05$)

With respect to the root biomass, significant differences were also found among the different treatments ($F=64.86$ $p < 0.001$). The H20% control seedling and the D1/3 and D1/4 treatments presented the highest values (0.393 ± 0.025, 0.383± 0.018 and 0.414± 0.025 g DM, respectively). In contrast, the H5% control and the F1 treatment presented the lowest values for root biomass (0.111 ± 0.006 and 0.089 ± 0.006 g DM, respectively) (Figure 1C).

The F1 and F2 treatment seedling presented a significantly lower root/shoot ratio (0.387 ± 0.025 and 0.574 ± 0.033, respectively) than the rest of the treatments (1.337-1.955) ($F=22.23$, $p < 0.01$) (Figure 1B).

The results showed that the use of OMSW digestate did not have any negative effect on the growth of *Lolium rigidum*; indeed, with certain treatments a certain positive effect was observed through the increase in total biomass (D1/3 and D1/4); while treatments F1 and F2 showed a positive effect on the growth of the biomass of the shoot of the seedling. In the treatments F1 and especially F2, the seedling contributed significantly more shoot biomass than root biomass, increasing the amount of part available for animal consumption. Although the root/shoot ratio is dependent on the specie and is defined during the ontogeny of the seedling, there are studies that show that it is strongly linked to external factors (Lynch et al., 2012). In previous studies the results showed that root growth is greater when the seedling has limited water or

nutrients (Kataki et al., 2019). In the tests carried out in this experiment, D1/3 and D1/4 treatments are the ones that presented the highest root biomass, so fertirrigation treatments (F1 and F2) are the ones with the most bioavailable nutrients, limiting the growth of the root and helping the growth of the shoot. Nutrients availability is one of the main factors that affect the root elongation (Dechassa et al., 2003), with the increase in nitrogen supply being the factor that most affects the root/shoot ratio. By increasing the nitrogen supply, the shoot increases in relation to the root of the seedling (lower root/shoot ratio) (Lynch et al., 2012).

3.4. Effect of OMSW digestate on *Lolium rigidum g*as exchange and nutrients

No treatment showed visual signs of injury to the seedling and, in general, the seedling seemed as vigorous as those grown in inorganic fertilizer (H5% and H20%).

In terms of the net photosynthesis rate, the results showed significant differences among treatments ($F=62.98$; $p < 0.001$); seedlings grown in the H5% control presented significantly lower values for A (1.2 ± 0.07 μmol m^{-2} s^{-1}) and seedlings grown in F2 treatment significantly higher values (12.5 ± 0.81 μmol m^{-2} s^{-1}). The rest of treatments showed similar values for photosynthesis

rate (around 6-7 μmol m^{-2} s^{-1}); no significant differences were found among them (Figure 2A).

With respect to stomatal conductance (Gs) values, no significant differences were found among the treatments ($p > 0.005$), with registered values of around 170 μmol m^{-2} s^{-1} (Figure 2B).

Intercellular CO_2 concentration (Ci) showed a similar trend to photosynthesis rate ; significant differences were found among treatments ($F=16.09$; $p < 0.001$); seedlings grown in H5% control presented significantly higher values for Ci and grown in the F2 treatment showed significantly lower values. No significant differences were found in the rest of the treatments (Figure 2C). Certain macronutrients such as nitrogen and Mg^{2+} have an important role in photosynthesis function (Mancilla-Leyton et al., 2012). Nitrogen is essential for the proper development of the seedling, and nitrogen deficiencies affect from growth and development to metabolism to resource allocation. On the other hand, Mg^{2+} plays a key role in the modulation of Ribulose-1,5-bisphosphate carboxylase/oxygenase in the stroma of chloroplast (Mancilla-Leyton et al., 2012).

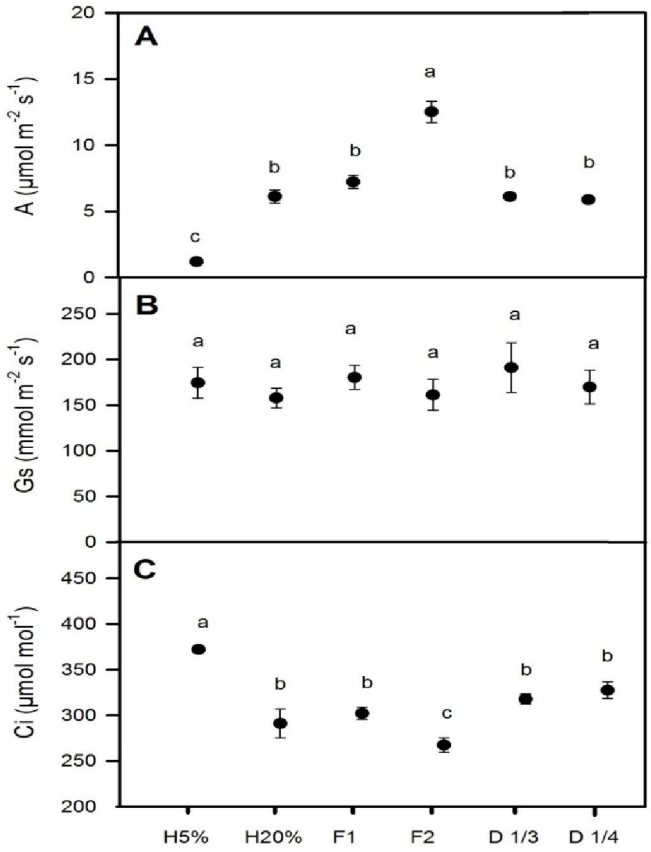

Figure 2. Net photosynthetic rate (A), stomatal conductance, Gs (B), and intercellular CO_2 concentration, Ci (C) in randomly selected fully developed leaves of *Lolium rigidum* var. *Wimmera* grown in the different OMSW digestate treatments. Where D: raw digestate for amendment of a nutrient-poor soil, D1/3: mixture 25% D-75 % silica and D1/4: mixture 20% D-80% silica, respectively. H5% and H20% are Hoagland nutrient solution at 5% and 20% concentration, respectively, and F1 and F2 are the liquid fraction of digestate used as fertilizer with one and two fertirrigates, respectively. Values represent mean ± standard error.

Although the Mg^{2+} content in the shoot of *Lolium rigidum* in the different treatments (0.29, 0.25, 0.19 and 0.16% for D1/3, D1/4, F1 and F2, respectively) was lower than that found in the shoot of the control seedling (H5% and H20%-, 0.28 and 0.36%, respectively) (Table 4), no decrease in the photosynthetic rate was detected. As far as nitrogen is concerned, the *Lolium rigidum* control seedling (H5% and H20%) presented a nitrogen Kjeldahl content of 0.76 and 1.29%, respectively. Similar values for H20% control nitrogen content were observed in treatments D1/3, D1/4 and F1 (1.04, 1.06 and 1.49%, respectively). In contrast, during the F2 treatment, the nitrogen content of the *Lolium rigidum* leaves increased to 4.02%. In *Lolium* roots, similar nitrogen values were found in the controls H5% and H20% (0.48 and 0.51%, respectively). Also, similar values for nitrogen content were found in the roots after treatments D1/4, D1/3 and F1 (0.36, 0.41 and 0.55%, respectively). The percentage of nitrogen content in the roots of ryegrass was higher after the F2 treatment (1.63%) (Table 4).

Nitrogen acquisition is one of the most important factors for seedling production. Nitrogen availability to animals is predominantly from forage proteins. Thomas et al. (2010) showed that sheep preferred to eat high-nitrogen seedling at the reproductive stage with higher nutritional value. Nitrogen is the main nutrient for proteins providing, which are required by animals for the production of milk and meat (Boland et al., 2013).

The availability of nitrogen for the development of forages from soil amendments or fertigation from organic fertilizers is an environmental advantage.

Table 4 Concentration of nutrients in the shoots and roots of *Lolium rigidum* var. *Wimmera* grown in the different OMSW digestate treatments. Where D: raw digestate for amendment of a nutrient-poor soil, D1/3: mixture 25% D-75 % silica and D1/4: mixture 20% D-80% silica, respectively. H5% and H20% are Hoagland nutrient solution at 5% and 20%, respectively, and F1 and F2 are the liquid fraction of digestate used as fertilizer with one and two fertirrigates, respectively OMSW: olive mill solid waste and NKjeldahl: Kjeldahl nitrogen.

OMSW digestate treatments	*Lolium* part	**Ca %**	**Fe mg kg^{-1}**	**K %**	**Li mg kg^{-1}**	**Mg %**	**Na %**	**P %**	**Sr mg kg^{-1}**	**N Kjeldahl %**
D1/3	shoot	0.83	204.5	2.15	1.38	0.29	0.64	0.30	21.48	1.04
D1/4	shoot	0.65	272.3	2.29	1.42	0.25	0.67	0.38	18.04	1.06
F1	shoot	0.38	207.5	2.69	1.23	0.19	1.16	0.25	11.85	1.49
F2	shoot	0.32	400.8	3.08	1.36	0.16	1.36	0.50	9.00	4.02
H20%	shoot	0.66	254.4	1.87	0.47	0.36	0.48	0.21	14.98	1.29
H5%	shoot	0.75	156.9	1.26	0.62	0.28	0.44	0.11	22.69	0.76
D1/3	root	0.37	2462.3	0.60	1.41	0.11	0.77	0.10	10.29	0.41
D1/4	root	0.33	1936.4	0.52	1.36	0.09	0.57	0.09	9.70	0.36
F1	root	0.35	2520.5	0.96	1.83	0.10	0.85	0.16	14.53	0.55
F2	root	0.63	6545.2	1.60	1.21	0.14	1.21	0.53	32.23	1.63
H20%	root	0.52	1267.3	0.61	0.87	0.23	0.47	0.09	9.94	0.51
H5%	root	0.32	917.4	0.86	1.00	0.21	0.90	0.09	9.25	0.49

Conclusions

This study evaluated the use of olive mill solid waste digestate as an organic amendment and as fertilizer. The results showed that the OMSW digestate contains: organic matter, macro and micronutrients which are suitable for use in agriculture as an organic amendment and as fertilizer. Heavy metal contents were below the threshold established by the Spanish and European legislation on sludge spreading. No pathogens were detected in the OMSW digestate. A clear delay in *Lolium rigidum* seed emergence was detected using the solid fraction of the OMSW digestate, but no phytoinhibition was observed with the other treatments. The most bioavailable organic matter remains in the liquid fraction of the digestate, improving the growth of *Lolium rigidum*. The results clearly showed that the F2 treatment (two fertirrigations of the liquid fraction of the digestate) was the one that best contributed to the development of *Lolium rigidum*, improving the growth of the aerial biomass, photosynthetic rate and nutritional content of the seedling.

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Conclusiones

1. El pre-tratamiento hidrotérmico suave a 121 ºC, 1,1 bar durante 30 minutos (A1) aumentó el rendimiento de metano del alperujo en un 36% comparado con el rendimiento del alperujo sin pretratar. El pre-tratamiento hidrotérmico suave rompió las fibras del alperujo por la mitad, tanto en longitud como en diámetro, ayudando a solubilizar los azúcares en forma de polisacáridos. El pre-tratamiento también ayudó a solubilizar compuestos fenólicos, alcanzando altas concentraciones de éstos compuestos de interés (hidroxitirosol, 658.9 ± 0.8 mg/kg, y dihidroxifenilglicol, 405.4 ± 0.4 mg/kg). Se ha demostrado que la solubilización de ciertos fenoles en los rangos de concentración obtenidos, no son inhibitorios para el proceso de digestión anaerobia. En cambio, otros fenoles como el tirosol, en concentraciones superiores a 198 mg/kg, se ha comprobado que es inhibitorio para el proceso de digestión anaerobia, provocando una disminución en la producción de metano.

2. Los resultados obtenidos en los experimentos llevados a cabo en esta tesis confirman el poder de la co-digestión anerobia de un substrato rico en carbono como el alperujo y otro rico en nitrógeno como son las microalgas. Durante la co-digestion anaerobia se mejoró la biodegradabilidad de ambos sustratos y la tasa de conversión. La co-digestión anaerobia también ayudó a aportar estabilidad al sistema, la biomasa algal proporcionó nitrógeno al sistema, equilibrando la relación C/N y aumentó la capacidad tampón del proceso.

3. Se ha demostrado que la pared celular de la microalga *Chlamydomonas reinhardtii* 6145, compuesta principalmente de proteínas unidas por enlaces covalentes, no tuvo ningún efecto negativo durante su co-digestión anaeróbica con alperujo. La producción de metano obtenida durante la co-digestión de *Chlamydomonas reinhardtii* 6145, microalga con pared celular, con alperujo fue mejor que la obtenida

durante la co-digestión de *Chlamydomonas reinhardtii* cw15, la microalga mutante sin pared celular, con alperujo.

4. Las principales diferencias entre la digestión anaerobia del alperujo, del alperujo pretratado térmicamente y del alperujo co-digerido con la microalga *Dunaliella salina*, estriba en que al introducir la microalga en el sistema, se mejora el rendimiento en metano, manteniendo concentraciones relativamente constantes de materia orgánica soluble, reduciéndose la acumulación de ácidos grasos volátiles.

5. La Función de Transferencia de primer orden y el modelo de Gompertz modificados mostraron una buena ajuste con los resultados experimentales obtenidos en esta Tesis ayudando a describir la cinética de la digestión anaeróbica del alperujo, del alperujo pretratado térmicamente y de las distintas co-digestiones alperujo-microalga estudiadas.

6. La microalga *Raphidocelis subcapitata* es capaz de crecer en aguas de lavado de aceite de oliva diluida con agua de lavado de aceitunas. Los dos efluentes líquidos resultantes del proceso de elaboración de aceite de oliva por centrifugación en dos fases. Las microalgas eliminaron materia orgánica de los efluentes, fosfato y nitrato. Además la co-digestión anaerobia de ésta biomasa algal obtenida con el alperujo, 75% alperujo-25% microalga, aumentó en un 7,0% el rendimiento de metano obtenido, comparado con la digestión anaeróbica del alperujo por sí solo. La utilización de los subproductos planteada ayuda a cerrar el ciclo de elaboración de aceite de oliva por centrifugación en dos fases, utilizando todos y cada uno de los subproductos generados en el proceso, y abogando por el aprovechamiento de todos los recursos y la mejora de la gestión ambiental.

7. El digestato resultante de la digestión anaerobia del alperujo, tiene un pH, conductividad eléctrica y concentraciones de nitrógeno óptimas para su reutilización como fertilizante y

como enmienda orgánica de suelos. No acumula metales pesados tóxicos ni tiene presencia de patógenos como *E. coli*, *Tenias* o *Salmonella*. El digestato del alperujo proporcionó nutrientes a la planta herbácea *Lolium rigidum*, ayudando a mejorar su contenido nutricional, aumentando la biomasa de sus brotes y mejorando su tasa fotosintética.

Conclusions

1. The Soft hydrothermal pre-treatment at121 ºC, 1.1 bar for 30 minutes of exposure time (A1) increased the methane yield of the pre-treated olive mill solid waste (OMSW) by 36% compared to the value obtained for untreated OMSW. The Soft hydrothermal pre-treatment helped to break the OMSW fiber in half both in length and in diameter, helping to solubilize sugars in the form of polysaccharides. The pre-treatment also helped to solubilize phenolic compounds achieving high concentrations of valuable compounds such as hydroxytyrosol, 658.9±0.8 mg/kg, and dihydroxyphenylglycol, 405.4±0.4 mg/kg, moreover, some of them being non harmful for the anaerobic digestion process at the concentration ranges tested. However, it was found that Tyrosol concentrations higher than 198 mg/kg were inhibitory for the anaerobic digestion process, bringing about a decrease in methane production.

2. These results confirmed the powerfulness of the co-digestion of the carbon-rich OMSW with nitrogen-rich microalgae. Co-digestion increased the biodegradability of both substrates and the conversion rate. Co-digestion also helped the stability of the anaerobic digestion system. The microalgae supplied nitrogen to the system, thus balancing the C/N ratio and providing extra alkalinity.

3. It has been demonstrated that the cell wall of the microalga *Chlamydomonas reinhardtii* 6145, mainly composed of proteins linked by covalent bonds, had no negative effect during its anaerobic co-digestion with OMSW. The methane production obtained during the co-digestion of *Chlamydomonas reinhardtii* 6145, microalga with cell wall, with OMSW was better than that obtained during the co-digestion of *Chlamydomonas cw15*, the mutant microalga without cell wall, with OMSW.

4. The main differences between anaerobic digestion of

OMSW, thermally pretreated OMSW and OMSW co-digested with microalga *Dunaliella salina,* were that the introduction of the microalga *Dunaliella salina* improved OMSW digestion performance, maintaining relatively constant soluble organic matter concentrations and reducing the volatile fatty acids accumulation. The co-digestion with *Dunaliella salina* reached the highest methane yield in comparison with the single OMSW and the thermally pretreated OMSW.

5. The first-order, Transference function and the modified Gompertz models showed a good fitness to the experimental results of this Thesis and could describe the kinetics of anaerobic digestion of OMSW, thermally pretreated OMSW and OMSW co-digested with microalgae.

6. The microalga *Raphidocelis subcapitata* is capable of growing in olive washing waters diluted with olive oil washing water from the two-phase olive oil manufacturing

process and has the potential to remove organic carbon and nutrients (phosphate and nitrate). The anaerobic co-digestion 75% OMSW – 25% *Raphidocelis subcapitata* increased by 7.0% the methane yield compared to the anaerobic digestion of 100% OMSW. The waste utilization raised, is an attempt to close the loop of the two phase olive oil production system, using each one of the wastewaters and wastes generated in the olive oil elaboration process and advocating for resource efficiency and environmental management.

7. The OMSW digestate, obtained after OMSW anaerobic digestion, has a pH, electrical conductivity and nitrogen concentrations optimum for its reuse as fertilizer and soil amendment. No toxic heavy metal accumulations and no presence of pathogens such as *E. coli*, *Taenia* or *Salmonella* were detected. The OMSW digestate provided nutrients to the herbaceous *Lolium rigidum*, helping to improve its nutritional content, increasing its shoot biomass and improving its photosynthetic rate.